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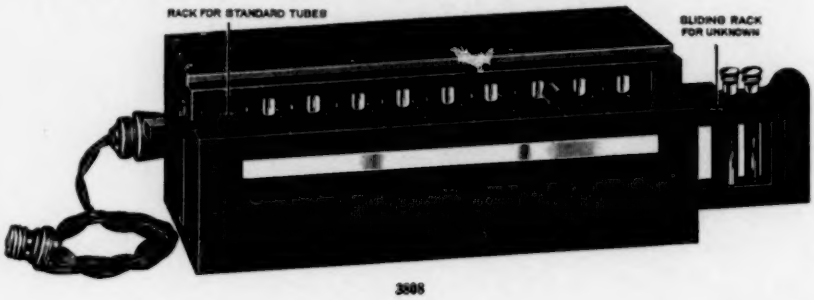

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STUDIES IN MICROBIC HEREDITY

VI. THE INFECTIVE AND TAXONOMIC SIGNIFICANCE OF A NEWLY DESCRIBED ASCOSPORE STAGE FOR THE FUNGI OF BLASTOMYCOSIS[1]

RALPH R. MELLON

From the Department of Laboratories, Highland Hospital, Rochester, New York

Received for publication December 21, 1925

The observations to be reported deal with the evidence of an ascospore stage for the simpler types of the group of fungi that are regarded as etiologic for the lesions of blastomycosis. Other forms of special resistance such as the so-called "dauernzellen" have also been observed. The demonstration of asci for this group has a distinct bearing on their rather uncertain taxonomic position and in addition offers an explanation for the noteworthy chronicity of the disease as it occurs in man. Furthermore, it may help to explain the recrudescences of the disease after apparently yielding to treatment. A preliminary note dealing with this observation has already appeared (Mellon, 1924).

The observations of Ricketts (1901) made him favorable to the possibility of ascospore formation but, with the exception of his third or higher group, he could not convince himself that such occurred. Even with this third group he observed what appeared to be terminal ascospores only occasionally and there was some question of their nature. He apparently has not

[1] From the standpoint of the mycologist the term "blastomycosis" or "blastomycete" may be open to considerable objection on account of its taxonomic ambiguity; yet in order to link up this study with a disease that clinically and pathologically is a distinct entity I shall refer to it in this paper by its usual name so as not to confuse those in medical biology who know it by no other name except, perhaps, oidiomycosis. This latter is not a taxonomic improvement on blastomycosis. If I were to suggest a name on the basis of this study it would be some species of the genus endomyces. Certainly from the mycologic point of view blastomyces is too general a term.

included any such among his photographs. His large forms, which contained very numerous intracellular bodies, do not constitute in his judgment sufficient evidence of endosporulation. The lack of a double contoured membrane for the inclosed bodies, their relative inability to stain, and their great number, are points which fail to correspond with the observations which we have made. The typical morphology of our bodies, combined with the prolonged viability of the cultures in which they are found, leaves no reasonable doubt that they are true ascospores.

Other writers, notably LeCount and Myers (1907), Stober (1914) and Wade (1916), describe very numerous, rather minute forms in the tissues of the host which suggest an endosporulation. They also find an occasional empty shell in the tissue, which LeCount and Myers believe supports the endosporulation theory; but Wade interprets such formations as the result of tissue activity and not as the product of vital activity on the part of the parasite. Furthermore, the small size of the parasite results, in his opinion, from a variation of the gemmation process, which he speaks of as a "frantic multiplication."

Although Wade's view of the origin of the minute forms is more probable, our cultural studies will show that LeCount and Myers' interpretation of the shell-like capsules is deserving of consideration. True ascus formation occurring in our cultures has not yielded many minute forms, but only structures containing four normal sized cells.

Furthermore, the direct observations of Hamburger (1907) show that although minute forms originate by rupture of a large cell they are not viable, and, properly, as I believe, he does not regard them as the result of ascus formation. Whether it is possible for minute germination units to be formed by a type of endosporulation not now recognized among these fungi is of course a question that only the future may decide.

It is to be recalled that Ophüls (1905) has shown that the parasites of coccidoidal granuloma—the blastomycotic disease occurring on the Pacific Coast—reproduce rather characteristically by endosporulation in the tissues. This feature, constituting one of the chief differences from the eastern cases, may

come to be regarded as one of degree only, in light of the observations to be here reported.

SOURCE OF CULTURES

The organisms studied corresponded to Types I and II of Ricketts. Type I proliferated chiefly by budding, although mycelia sometimes developed as the cultures aged. In Type II the mycelial element initiated the growth of the organism by fastening itself to the media and from this a profusion of buds quickly developed. Mycelial arborizations frequently penetrated the medium. Both these cultures were isolated from the lesions of the same patient.

Type I was easily recovered in purity from the young lesions (fig. 1, at *a*), while Type II came invariably from the older lesions where its isolation was effected with much difficulty. This double isolation may be of significance, suggesting that the conflict between host and parasite evolved a different type in the older lesions. The possibilities of this important genetic consideration has been made the subject of experiments whose results will be reported in a separate communication.

Figure 1 shows the lesions about the face of the case from which the organisms were isolated. Similar lesions were found on the arms and legs. From the young lesions at *a* a Type I yeast was obtained. The lesion was closed and was surgically removed *in toto* under aseptic conditions. From the border of an older lesion on the leg Type II was isolated, but Type I did not grow; yet from a young lesion not over a month old and removed only a few inches from the older one, Type I was readily isolated. Clinically and histologically these lesions were typical of the disease. The recognition of the asci in the cultures was in essence a by-product of an inquiry into the biologic nature of secondary colonies among the bacteria.

It was desired to study at first hand these secondary growths among fungi, where they are common, with the idea of correlating their significance if possible with similar developments among the bacteria. It is rather generally recognized that these secondary colonies among the fungi represent a distinct

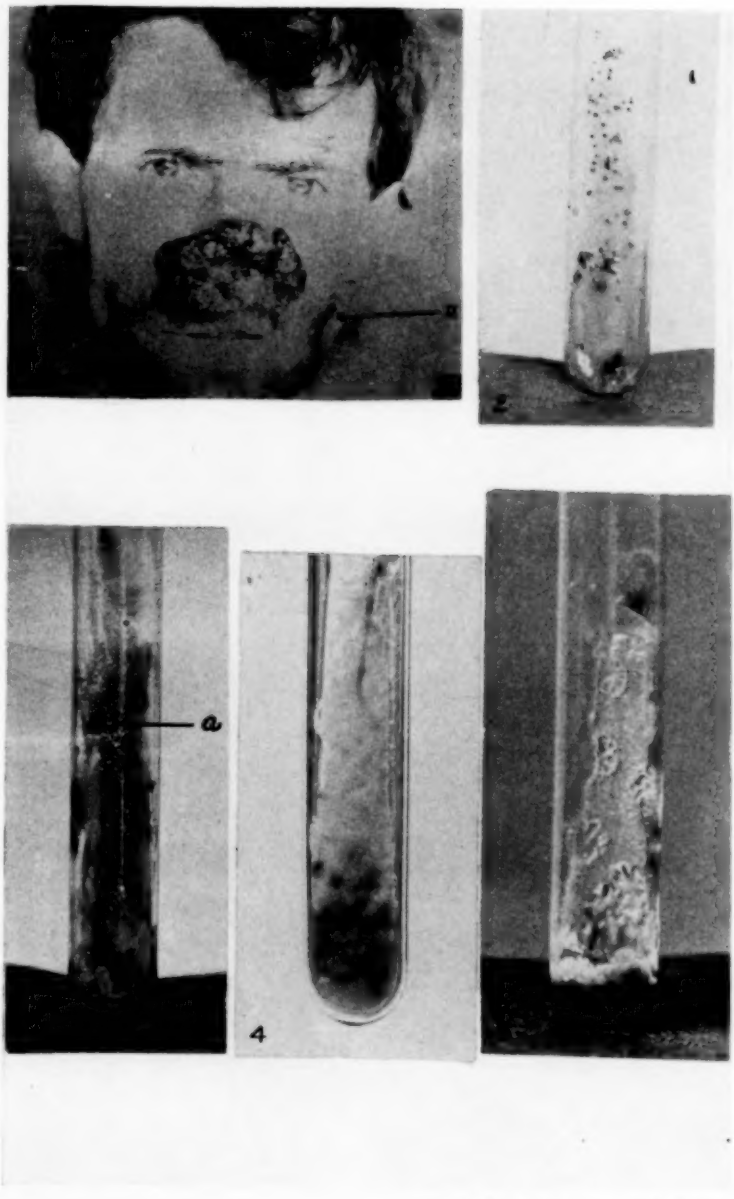
phase in the life history of the fungus, and one of our previous studies on variability (Mellon, 1922) made it appear that they have here the same significance.

It was in these secondary colonies exclusively that the forms of special resistance such as ascospores, oidial forms, chlamydospores and "dauernzellen" were found, and the fact that their development was often a matter of weeks or even months may explain why asci have not been observed before. Most of our studies were conducted on Type II where the secondary colonies showed to special advantage and were readily recognized as pigmented elevations springing from a background represented by the primary growth. Their pigment was usually black or very dark brown but in some instances the secondary colonies were buff, or even pink in color.

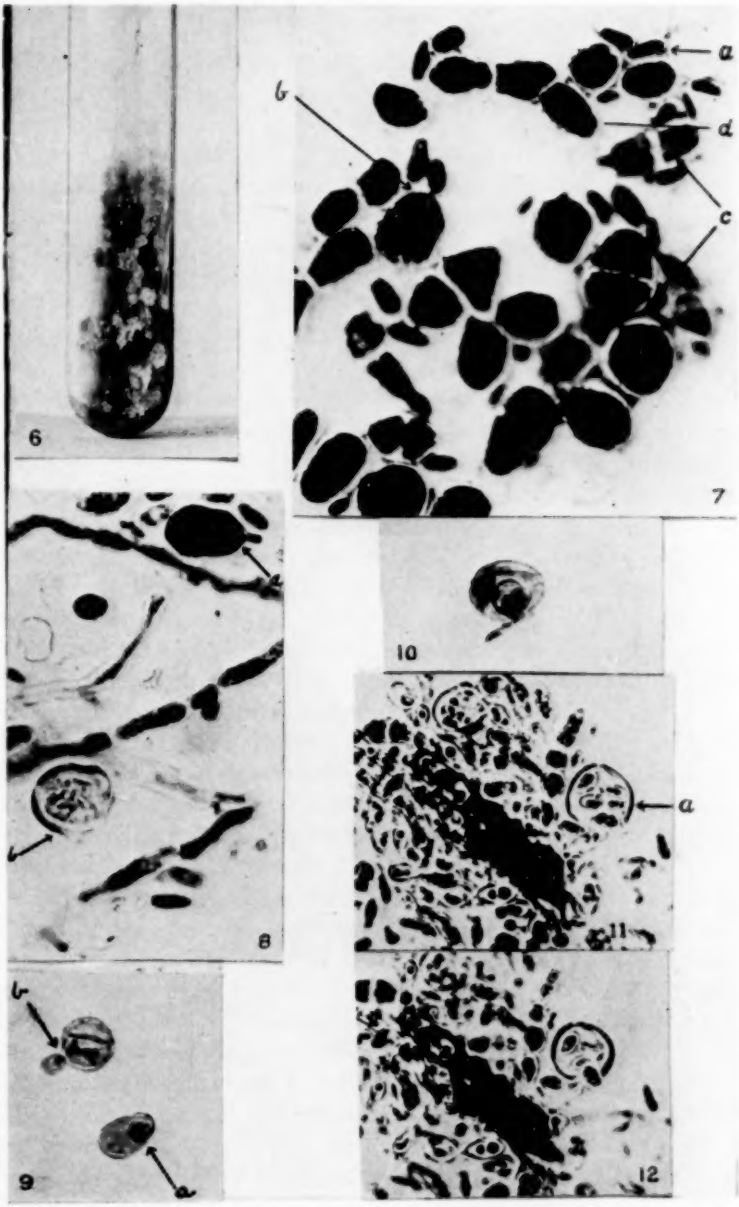
Figure 2 shows to good advantage the black colonies on a background composed of the relatively colorless primary growth. Figure 3 shows a chagreened secondary type of growth with the black secondary development at *a*. Figure 4 shows at the bottom of the tube the penetration into the media's depths of beautiful dendritic arborizations, taking origin apparently from the same structures as the black secondary colonies on the surface. These are the intermediate or Type II fungi. Figure 5 shows the secondary developments of Type I. The ulcerated or pock marked looking areas are tough in consistency, and have a pleomorphism, microscopically, which identifies them as developmental phases in the life cycle of the organism.

Figure 6 is a Type II organism showing clusters of colorless and pigmented secondary colonies. These colonies were usually very tough and could be removed *en masse* from the media. Microscopically they are usually dominated by the oidial form, so-called, often pigmented, which occurs singly or in threads or pairs. They are usually much larger and differ in shape from the normal forms of the organism. They have also a much thicker capsule and stain with great intensity.

These morphologic differences are clearly seen in figure 7 where the small oval organism at *a* represents the so-called "normal" form of the parasite. The oidial form may reproduce



(Mellon: Studies in Microbic Heredity)



(Mellon: Studies in Microbic Heredity)

by budding as at *b* although the forms at *c* suggest a transverse fission. Subsequent divisions may form a thread, the beginning of which is seen at *d* in figure 7 and also in figure 21. Although the transverse division wall between these cells suggests pure fission, the cell appears primarily to put out a bud, which is then separated from the maternal cell by a transverse division wall. Although finality on this point awaits further direct study of the germinating forms, we have seen no evidence that these "oidia" arise by fragmentation of a mycelial filament as some authors believe.

In figure 8 the mycelial phase is observed in addition to an oidial cell at *a* showing early budding, and an encapsulated form at *b* whose possible significance will be considered later. In figure 9 (Type I) we have what corresponds to the "dauernzellen" of H. Will (1895). In figure 9 the nucleus-like structure seen at *a* is apparently capable of undergoing typical gemmation as seen at *b*. Note the transverse rent in the capsule. When the large intensely staining oidial forms germinate they may put out from one to four or five of these buds.

In figure 10 one sees a doubly contoured form of the organism within a large form, a rent in whose capsule is indicated by the triangular refraction lines inclosing it, and by the stripping of the capsule itself. This form is structurally different from the four-celled ascus characteristic for this species. It is probable that this preformed cell is destined to bud forth from the maternal cell as an oidial stage, further stages in which process are detailed in connection with figures 18, 20, 21 and 23.

The secondary colonies which are red or buff color usually consist of these rather large forms having a transverse rent in their capsule as if something had escaped. The edges are frequently folded back. In contradistinction to the black secondary colonies the red colonies may not be viable. If they contain well stained forms, however, they are always viable.

The black colonies are noted for their great viability—in fact if it were not for their development we should have long since lost this culture which has now been under observation for five years. Under conditions where only the normal forms develop,

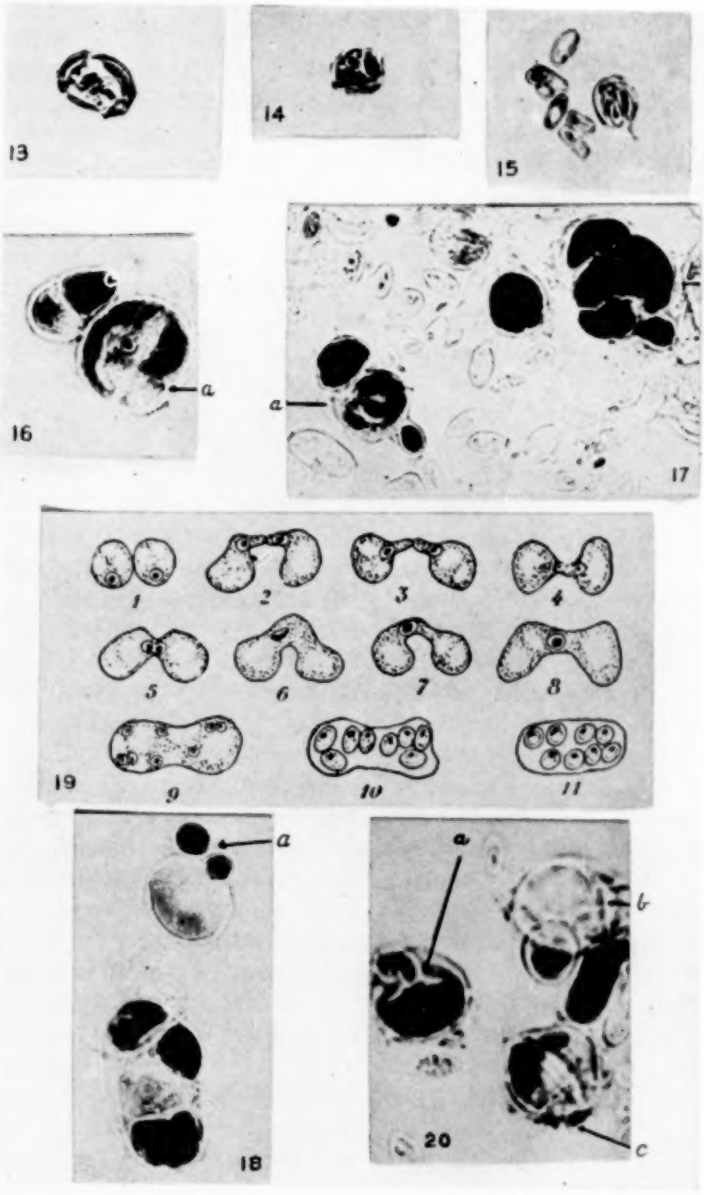
the cultures' average viability is about a month or less, but under conditions where these secondary black colonies develop the viability has been extended for as long as two and a half years.

Under these conditions the primary growth constituting the normal form of the organisms does not stain, while the intense staining of the pigmented forms is usually a safe indication of their viability. Occasionally, however, even they may undergo a progressive fatty type of degeneration as often seen in the dead normal forms, and when in this stage they are usually poorly staining and non-viable.

Here we have a condition of affairs that in its bearing on the nature of "involution forms" with bacteria is highly significant. We see that these oidia and related forms of special resistance are entirely comparable to what the bacteriologist is pleased to term involution forms. In fact if we were to look at these fungi with a low power microscope the morphologic resemblance of *itself* would suggest a pleomorphic bacterium; but the analogy extends much further than that. Just as with the bacteria we find that these pleomorphic forms of special resistance are often segregated in secondary colonies. Furthermore they are possessed of modifications in the reproductive mechanism, and—as we shall show in another paper—may be the *anlage* for the origin of variants within pure line cultures. Thus the analogy is complete, constituting rather formidable evidence for our contention *that biologically bacteria may be properly regarded as fungi which have been telescoped down into a state of existence where their life cycles, although much compressed and often abbreviated, are still not obliterated.* It would seem that it is largely in this sense that bacteria can be regarded as "simpler" forms.

ASCOSPORE FORMATION AND OTHER FORMS OF SPECIAL RESISTANCE

It is clear then that the secondary colonies of these fungi are the expression of a distinct evolutionary phase in the life history of the parasite. Since the ascospore is the "perfect stage" of the mycologist, representing as it does the culmination of the



(Mellon: Studies in Microbic Heredity)

reorganization activities, it would be natural to search for this stage in the secondary colonies, and in this our expectations were not disappointed.

It was in a secondary buff colored colony grown on non-peptone media where we found the forms about to be described. They were first observed after the culture had been grown for about two months at room temperature. In figure 11, at *a* is seen clearly an ascospore containing four normal sized, normal staining, doubly contoured forms of the parasite. The wall of the ascus at one point has almost disappeared, and in figure 12, which is the same picture taken about three hours later, one of the cells in this ascus has changed its position to a right angle and can be seen emerging from the cell. Note, too, the contraction of the ascus as the cell is forced out. This decreased size is not related to the slight change of focus necessitated; the magnification is precisely the same. The organisms were placed in a closed cell containing broth and lightly stained in thionin.

The intensely staining, diagonally located structure is an oidial filament whose details are obscured by the pigment. Note, too, that many of the cells contain circular, rather highly refringent bodies which have often been mistaken for spores, but are not doubly contoured, stain poorly if at all, and vary greatly in size. They are probably nothing but fatty granules since such structures are usually non-viable.

In figure 13 is another four-celled ascus where the definition of the individual cells is sacrificed to show the rupture points in the extraordinarily thick sclerotic capsule. In figure 14 is a ruptured ascus containing three cells; the fourth may have escaped. Figure 15 shows another ruptured ascus containing two cells. Note the frayed border of the capsule near the point for emergence of the entirely normal appearing vegetative cells. Note also that the chromatin in the free cells with one exception stains poorly.

In the large form of figure 16 is a small circular central body which may be a nucleus; a smaller one is seen at *a* in the other half of the cell, which shows a transverse line of division so frequent with these forms. In figure 17 at *a* is a similar form ap-

parently budding, as is the larger intensely staining form at *b*. Figure 18 at *a* shows early budding of a large oidial or dauern cell that may yield a circular form of the organism—the normal form being oval.

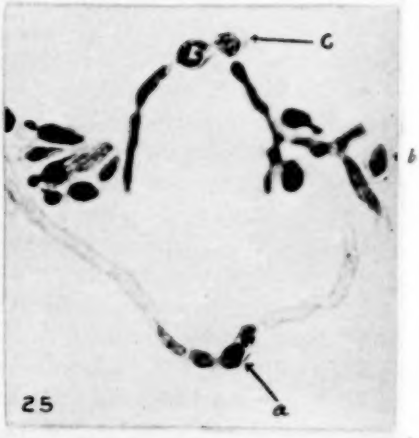
It is possible that some of these large nucleated forms are gametes inasmuch as copulation among the yeasts usually precedes the formation of the zygospore, which in turn *becomes* the ascospore. Such interpretation is entirely in harmony with the newer knowledge of the biology of yeasts as indicated in Guillermond and Tanner's book (1920). Figure 19, taken from this book, shows the nucleus and its rôle in copulation and ascus formation.

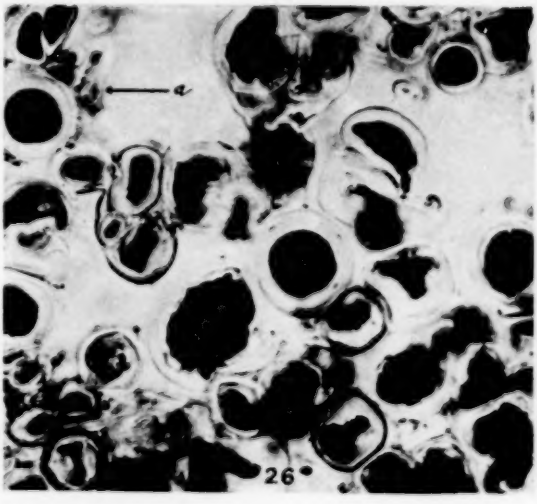
Although it is difficult to say whether the nucleus-like structure described by Ricketts (loc. cit.) is the same that we have seen, his suggestion as to its possible rôle in reproduction is pertinent. His descriptions recall the type of structure seen commonly in the "dauernzellen" which he did not recognize as such; the work of H. Will in 1895 may not have come to his attention.

GEMMATION WITH CAPSULAR DEHISCENSE

This rather interesting and unusual method of germination as suggested by figure 20 may explain in part the large number of empty cells seen in the pink or buff colored colonies. In contradistinction to the frequent non-viability of these colonies this particular one was quite viable.

In figure 20 at *a* it appears that we have a bud forcing itself through a transverse rent in the cell. Although still connected with the maternal cell, the semi-circular separation between the chromatic masses presages the formation of a transverse division wall such as characterizes an oidial filament as it springs from a maternal cell (figure 21 at *a*). At *b* of figure 20 the process appears to be somewhat further advanced since the bud, although attached to the capsular "lip" reflected from the rent, is definitely demarcated from the inner contents. The form at *c* suggests that dehiscense of the capsule is complete, the bud having escaped through the rent in the cell leaving the residual chromatin.





(Mellon: Studies in Microbic Heredity)

It is entirely possible that the new bud may completely free itself before reproducing again, or that it may remain attached for a time as indicated in figure 21. Here is an oidial filament that appears to have arisen by budding through the capsule of the older form at *a*. The capsule of the older form is undoubtedly attached to the intensely staining filament. The impression of nuclear extrusion is also given by figure 22, but the absence of a thicker wall for the extruded portion makes the form difficult of interpretation.

Figure 23 suggests an early stage of the process before rupture of the capsule. Apparently a single well developed, doubly contoured, typical yeast is preformed within the membrane of the older cell whose capsule is quite thin at this point. This form probably represents a still earlier stage of figure 10, before capsular rupture.

Vuillemin has described internal globules, absolutely analogous in appearance to yeasts, which form on the interior of the filaments of the *Endomyces albicans* (Guillermond and Tanner, 1920). He considers them as resistant forms. Such forms among the fungi are known to bud into the intercalary units of mycelium and live parasitically on their protoplasm. This preformation of adult elements within protected phases of the parasite appears to be related to the forms just described by us.

Figure 24 at *a* shows two internal globules in a bulbar dilation of a filament (Type I). This is probably one form of chlamydospore, another stage of which is seen at *a* and *c* of figure 25, also a Type I fungus. At *b* is a free one. A most striking type of resistant form is seen in figure 26; not only are the forms unusually large and intensely staining but the capsules are enormously thickened. Note size and deficient staining of "normal" forms at *a*.

It is of special interest that these forms developed in the secondary black colonies of an agar slant containing 2 per cent NaCl. Although growth on this medium was scanty, these structures were readily viable when the culture was two and one-half years old, while the poorly staining normal forms in the primary growth were not viable. The high concentration of NaCl in

this medium recalls the conditions for the development of the well known "involution forms" of the plague bacillus on NaCl agar.

Without expressing an opinion as to the real nature of such forms with *B. pestis*, it should be perfectly clear that with this fungus the only *true involution forms* are the non-viable original or so-called *normal* forms of the parasite. The fact that this is often true for similar forms with the bacteria makes it inconceivable that they should continue to be regarded as they have been in the past. Vuillemin's *Cryptococcus lithogenes* has quite similar forms morphologically and physiologically, and he has given them the name of chronispores or chlamydospores.

CONDITIONS UNDER WHICH ASCOSPORE FORMATION WAS OBSERVED

Inasmuch as the asci have been observed in the buff colored or pink types of secondary colony described, it may not be out of place to indicate roughly the conditions under which the latter were observed. Inasmuch as the media in this laboratory are routinely buffered with disodium glycerophosphate as reported by us some time ago (Mellon, 1921) this salt was usually a component.

When 2 per cent sodium chloride was added to a medium buffered to a pH of 8.0 with glycerophosphate some of these pigmented colonies were observed to occur after several weeks in the ice chest, preliminary growth having been effected at room temperature as is always the case for this strain unless otherwise stated. They have also been observed to occur on Bordet's glycerin-potato medium, though it is rare for them to appear before a few weeks at least. The essential purity of these cultures was certified by their origin from a single cell and by the fact that the secondary colonies almost invariably gave rise to the original form of the organism.

More commonly we employed a veal infusion without peptone, buffered with sodium glycerophosphate to a pH of 6.0 by

titration with HCl. Three per cent (3 per cent) of glycerin or 1 per cent of maltose was added to this agar just before use.

I have got the impression that drying of the media facilitated the occurrence of these special growth forms. This is evidenced in two ways: First, by their occurrence near the upper part or the sides of the tube where the medium was slowly evaporating, and second, by their more frequent development on a medium which had evaporated from one-fourth to one-third of its volume before being somewhat diluted by the addition of the sugar solutions above mentioned.

Glycerin usually predisposes to a raised folded type of secondary growth which maltose is not so apt to give. Although it is on the latter type of medium that ascospore formation has been most constantly observed with this strain, I do not wish to give the impression that it did not occur at times on other media. For example, it was observed on ordinary agar containing peptone and buffered with the glycerophosphate salt to neutrality.

While thus detailing in a general way the conditions under which we have observed ascospore formation to take place we appreciate nevertheless that there are certain factors having to do with the strain itself that may frustrate, or perchance facilitate, the reproduction of this or other stages in the life cycle of an organism. We are distinctly of the impression that the descendants of these specialized forms of the secondary colonies *inherit* some of the adaptability of which the forms themselves are the expression.

This is shown, first, by the relative ease with which they may recapitulate the development of secondary colonies, independently it would seem of the media; secondly, by the fact that other pure lines which have *not* been recently descended from these reorganized forms often show no tendency to form secondary colonies, even on the identical media. But these considerations begin to involve the origin of differences within pure line strains of these fungi.

Inasmuch as the next paper of the series considers the biologic origin of the mould type (Type III) from our Type II it would seem appropriate to discuss the evidence for the view just ex-

pressed in this communication. Such occurrences explain why it is not always possible to formulate an environment that will evoke the desired stage in an organism's life cycle.

DOES ASCUS FORMATION OCCUR IN TISSUES OF HUMAN CASES?

I have referred to a work of Wade and those whom he quotes, dealing with the possibility of endosporulation in the host's tissues. Although Wade states that he observed no evidence of true endosporulation the cultural results which we have obtained suggest a different interpretation of the empty shell-like figures which he finds in the tissues. In his figure 8, which is reproduced as our figure 27, one observes a large body with a thick laminated capsule, apparently not intact, and whose contents are amorphous. This capsular formation is believed by Wade and certain European observers to be the direct result of the action of the tissues and not the result of the vital activity of the parasite.

In form and size the resemblance of these tissue structures to our ascospore cases opens up the possibility of their being a true ascus from which the cells have escaped. It is conceivable, too, that they represent abortive ascus formation. That the latter does occur is brought out by our figure 8 at *b* where a form of exactly the same proportions and morphology is encountered in a culture where no true ascospores could be made out with certainty; yet we usually find them associated with the deeply pigmented oidial forms as indeed the figure shows; so this form may represent an abortive attempt at ascus formation.

In respect to the origin of morphologic forms of parasites that are believed to be the result specifically of the influence of the host's tissues, I (1919) described years ago an in vitro club formation with a streptothrix that I regarded as homologous with these well known formations with actinomyces. Latterly Bayne-Jones (1925) has verified the observations with the true actinomyces showing that club formation occurs in plain agar in the test tube.

DISCUSSION

The bearing which these observations may have on the chronicity of the disease is obvious. It is a matter of common knowledge among those who have seen cases treated clinically with potassium iodide or x-ray, or both, that lesions may seem often to disappear entirely, only to break down at the same or at a distant location. These sac-like structures occurring in the cultures are highly resistant as indicated by their increased viability. If the similar forms present in the tissues are also highly resistant the clinical recrudescences may find explanation in their subsequent germination.

The cultures indicate that the new race so derived has been physiologically rejuvenated, which permits it to make a renewed assault on the host. The ready susceptibility to destruction of young forms by therapeutic agents would contrast with the relative invulnerability of these larger forms having the thick hyaline-sclerotic capsules which may make them fast, so to speak, to agents directed against them.

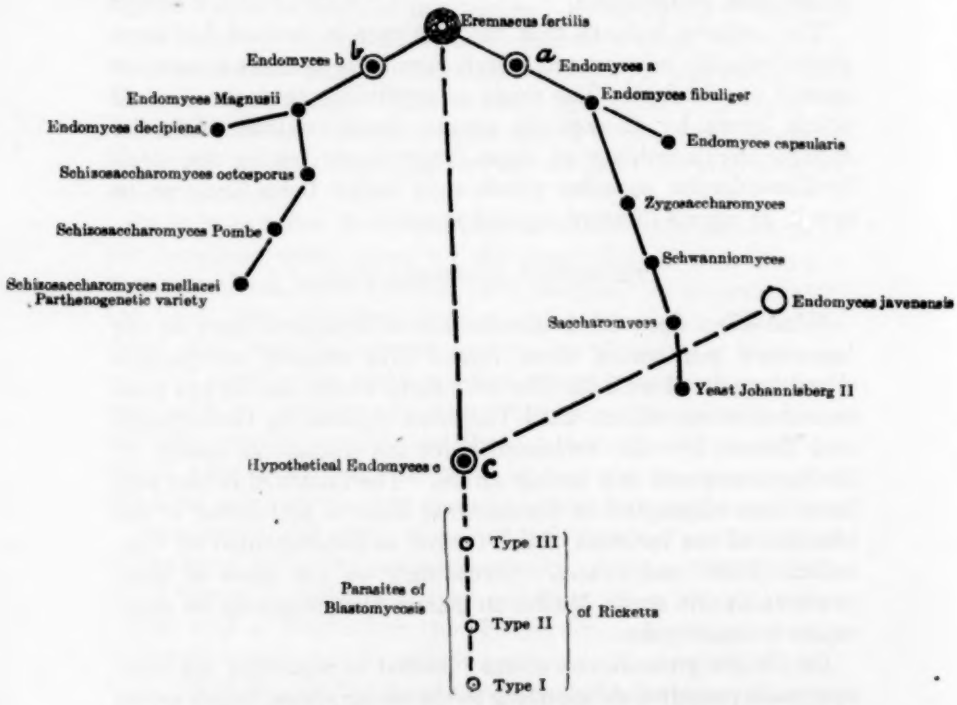
TAXONOMIC CONSIDERATIONS

What effect does the demonstration of ascospores have on the taxonomic position of these forms? An entirely comparable situation existed with the Thrush fungus which has always been regarded as an oidium until Vuillemin (quoted by Guillermond and Tanner, loc. cit.) reclaimed it for the endomyces family by finding ascospores in a certain strain. The failure of others who have since attempted to demonstrate them is attributed to the plurality of the varieties of this fungus as demonstrated by Castellani (1916) and others. Accordingly on the basis of their presence in one strain Vuillemin classes the fungus as an *endomyces* or ascomyces.

On similar grounds one seems justified in regarding the blastomycosis parasites as belonging to the ascomycetes, which would abrogate their inclusion among the oidia. Furthermore, their classification by Vuillemin as cryptococci—because no asci were observed—also becomes obsolete. Furthermore, the validity

of the oidium as a genus has gradually come into disfavor with mycologists.

According to Guillermond its general form is identical with the schizosaccharomycetes, a yeast reproducing largely by fission. Certain of our parasitic oidial forms appear to reproduce in this way (fig. 7). The diagram below taken from Guillermond and Tanner shows that the schizosaccharomyces are direct descendants of an endomyces which has an oidial phase in its life history, while the saccharomycetes or finely budding forms descend directly from an endomyces that has a budding instead of an oidial phase in its life history.



Now since the blastomycosis parasites exhibit conspicuously both budding and oidial phases arising from a mycelium that produces both asci and chlamydospores, it would appear that

they occupy an intermediate position between the lines of descent as diagramed above. The postulation of a third hypothetical endomyces *c* as shown in the diagram helps to clarify their phylogenetic relationships. The validity of such provisional arrangement is reinforced by the fact that the *Endomyces javenensis* can be successfully allocated in it.

This is a species existing in nature whose characters make its position transitional between endomyces and saccharomyces, much as the characteristics of the parasitic group make them transitional between saccharomyces and schizosaccharomyces. The conception of both these transitional groups arising from a transitional ancestor would not seem unnatural. Inasmuch as the organisms isolated from the Pacific Coast cases have not, to my knowledge, been shown to reproduce by budding it does not seem that they should be included in this group, but rather among the true molds.

CONCLUSION

1. Evidence is produced for the formation of four-celled asci with Types I and II of the parasites of blastomycosis as described by Ricketts. This *perfect stage*, according to mycologists, represents a form of endosporulation not definitely observed before.

2. This phase of the culture's development appears in our experience exclusively in the so-called secondary colonies, although all varieties of secondary colonies do not contain them. Associated with the asci are other forms of special viability such as the "dauernzellen," and oidia which may or may not be pigmented.

3. The probable occurrence of asci and related special growth forms in the tissues of the host offers plausible explanation for recrudescences of the disease after apparent cure.

4. Demonstration of ascus formation in the cultures suggests allocation of these organisms among the ascomycetes (endomyces) rather than with the oidia of Ricketts or the cryptococci of Vuillemin.

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STUDIES UPON BACTERIAL SPORES

I. THERMAL RESISTANCE AS AFFECTED BY AGE AND ENVIRONMENT

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INTRODUCTION

The resistance of bacterial spores to the destructive action of high temperatures has been a matter of general interest to bacteriologists for many years, but the significance of this resistance as a factor in practical home and industrial problems, particularly in the preservation of foods by canning, has but recently come to be appreciated.

The pioneer work of Russell (1895) thirty years ago, followed promptly by that of Prescott and Underwood (1897, 1898) established beyond question the relation of bacteria to food canning problems, and since that time numerous investigators have confirmed their conclusions and shown that successful food preservation is largely a problem of applied bacteriology. The findings of the early investigators were promptly evaluated and the processes involved in the canning of foods were modified to conform to the new information. Bacteriological investigations in this field were, however, for many years concerned primarily with the isolation and study of the microörganisms causing spoilage in canned foods, no thorough study being undertaken of the fundamental problems involved, particularly with respect to the thermal relations of bacterial spores.

Attention was forcibly drawn to this matter during the World War by heavy losses from valuable stocks of canned foods in warehouses, through spoilage, and particularly by outbreaks of

botulinus poisoning resulting from the use of insufficiently processed canned foods. Studies which these experiences initiated are still in progress.

For a thorough understanding of the problems involved in the present investigations, some familiarity with the observations of other workers upon bacterial spore resistance is essential. Sufficient space, however, is not available here for a detailed review of their reports, and so for the benefit of those who may care to look into the subject further, a condensed summary of their findings is appended at the end of this paper.

It is apparent that a number of influences are operative in determining the resistance of spores. In the reports of other workers it has been pointed out that the hydrogen-ion concentration of the medium in which the spores exist is one factor, and it has been suggested that the age of the spores may have something to do with their degree of resistance. One group of workers has made observations which indicate that possibly the amount of moisture present may be important, and that oil or other non-aqueous liquids may exert a protective influence. Others have observed that the kind of nutrient substances used in the cultivation of certain pathogenic forms apparently affects the resistance of the spores developing in the cultures, and the temperature of incubation has likewise been pointed out as an important factor.

Outside influences, however, have not been responsible for all the variations noted; for differences in resistance have been recorded where identical cultural conditions prevailed, and the opinion has been voiced that variation in resistance is an inherent property of spores which is not affected by conditions of environment.

It is seen, therefore, that the results of investigations by various workers are inconclusive, and further study of the subject is needed.[1] The matter is of immense practical importance as well as of considerable theoretical interest.

[1] Since the manuscript of this paper was completed the attention of the writer has been drawn to an important contribution to studies upon spore resistance by Esty and Williams (Jour. Infect. Dis., vol. 34, no. 5, p. 516–528), published

The problem of spore destruction is a complex one. The high resistance which many spores exhibit calls for rigorous treatment of the medium in which they are present to assure its sterilization. From the standpoint of the food canner this is a serious matter, for the processing required to destroy the troublesome spores often means injury to the appearance and quality of the canned product. Of particular significance, however, from both the practical and theoretical standpoints is the variation in resistance which they show. Not only do the spores of various species of bacteria show differences in their degree of resistance, but different strains of the same species often vary widely in this respect. Of still greater significance is the wide variation in resistance of the individual spores of a single strain. That these facts have an important bearing upon the occasional failure of hitherto successful practices in food canning can hardly be questioned; and until more is known concerning the factors which determine the resistance of bacterial spores there will always be uncertainty as to the outcome of particular canning procedures.

The investigations herein recorded were undertaken for the purpose of getting light upon certain phases of this important problem, and it is hoped that the results secured will not only be of assistance in removing much of the uncertainty that now accompanies food canning, but also help to a better understanding of the nature of bacterial spores.

EXPERIMENTAL PART

In preparation for a study of the factors affecting the variations in heat resistance of bacterial spores several matters had to be taken into consideration:

1. The selection of a suitable organism for the tests.
2. The development of a method that would assure maximum spore formation.

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just as the present investigations were being completed (May, 1924). It is a pleasure to note that although their investigations were headed in a slightly different direction, and different methods were employed in the determination of thermal death points, the results of the two pieces of work, so far as they cover the same ground, are mutually confirmatory.

3. The establishment of a definite point of departure for experiments through study of the spore cycle of the organism under observation.

4. Provision for the preparation and standardization of spore suspensions.

5. The development of a method for testing the thermal resistance of the spores.

THE ORGANISM

The organism selected for these studies was the *Bacillus mycoides*, one of the species of bacteria normally present in garden soil. It is commonly associated with the decomposition of organic matter and has frequently been isolated from canned foods. The strain of this organism used was one of 19 isolated from garden soils of 17 different states and was chosen because of the relatively high thermal resistance of its spores as shown by preliminary tests. This particular strain was isolated from Texas soil.

A number of characteristics of the *Bacillus mycoides* make it an especially favorable organism for studies of this sort: it is readily cultivated in the laboratory by ordinary laboratory technique, and grows rapidly; it forms spores readily and abundantly when proper environmental conditions exist; it has a striking and characteristic type of growth which facilitates the reading of tests and the detection of contamination; and it is non-pathogenic.

The morphological and physiological characteristics of the *Bacillus mycoides* are too well known to require consideration here. It is important to note, however, that an abundant oxygen supply is essential for spore formation, and that the optimum temperature for vegetative growth is 30°C.

MAXIMUM SPORE FORMATION

In the cultivation of *Bacillus mycoides* in the laboratory it is observed that spore formation takes place, most abundantly at least, at the surface of the medium. This being the case, provision for maximum aeration of the cultures in this work appeared necessary. Under laboratory cultivation it is also noted that this organism forms a thick felt-like scum or pellicle upon the surface

of liquid media, and a tough tenacious surface growth upon solid media, such as nutrient agar. So tough is this mass growth that it is impossible, except in very old cultures on solid media, to prepare an even suspension of the organism or its spores by ordinary agitation in liquid. To make thermal death point tests uniform suspensions of the spores are required. It was necessary, therefore, to provide for some means of separating the filaments or chains of cells at any stage in the development of the culture.

As a result of preliminary trials a method was developed which furnished ideal conditions for both the aeration of the cultures and the ready separation of the cells and spores as desired. The method was as follows:

Clean, fine quartz sand, such as is used in greenhouse experiments, was passed through a standard 40-mesh brass sieve, subjected to thorough washing, and then dried. After drying this was measured into the ordinary glass petri dishes, 15 by 100 mm. in dimensions, in 25-gram quantities, distributed to an even depth over the bottom of the plate, and then roasted in the dry air oven until completely sterile. This sand served as the substratum for the culture.

A satisfactory nutrient solution was next sought, and after repeated trials of different substances standard beef extract-peptone broth, made according to the formula recommended in the Manual of Methods for Pure Culture Study of Bacteria (1923) was selected as most suitable for the work.

In preparing these sand cultures a suspension of the spores from an old agar culture was made in a quantity of the sterilized nutrient broth, and brought to a boil in order to destroy all vegetative forms that might be present. With a sterile pipette, using aseptic precautions, just enough of this spore suspension was transferred to the sand plates exactly to saturate the sand, and the cultures thus prepared were placed in the incubator at 30°C.

It is seen that under these conditions the spores were suspended in a highly favorable nutrient medium, the fine particles of sand with their adsorbed layer of air assured maximum aeration, and the temperature of the incubator was the optimum growth temperature for this organism. Hourly examination by microscopic

technique of the grains of sand from these cultures early showed the chains of cells hugging closely the surfaces of the sand particles, and their very rapid development proved that optimum conditions for growth had been provided. Very prompt and abundant spore formation was likewise secured.

THE SPORE CYCLE

It was necessary at this point of the work to determine the length of the spore cycle of the organism under study in order that exact information might be had as to the age of the particular spores being used at any time, and especially that freshly formed spores might be subjected to thermal death point tests, so that a definite starting point for resistance studies might be established.

Accordingly, sand cultures were prepared from a suspension of spores in the manner described, and placed at 30°C. to incubate. At the end of one hour, and at hourly intervals thereafter, suspensions were prepared by shaking a small quantity of these sand cultures in tubes of sterile water. Microscopic examination was then made of these suspensions for evidence of vegetative growth and spore formation.

After preparation of specimens for microscopic examination a portion of the suspension was heated for one minute in a small tube immersed in boiling water and inoculations were made from this into nutrient broth. It was hoped in this way to determine the length of the germination period as well as to check upon the microscopic findings with respect to the length of the spore cycle. The cultural method failed to accomplish its purpose, however, as either the heating was not sufficiently long continued to destroy all vegetative cells or else the germination period of some of the spores equaled or exceeded the length of the spore cycle for other cells. The microscopic tests, however, proved satisfactory as they afforded an opportunity not only for determining the length of the spore cycle but also for observing the various stages passed through by the organism in the formation of spores.

The protocol of one of these experiments is given as follows:

Determination of length of spore cycle of Bacillus mycoides

Material: Sand cultures prepared by inoculating sterile sand with a suspension of the heat spores in nutrient broth.
Temperature of incubation: 30°C.
Date of test: November 26 and 27, 1923.
Microscopic findings:

TIME	INTERVAL	NOTES
	hours	
9:30 a.m.	At start	Spores only
10:30 a.m.	1	Spores only
11:30 a.m.	2	Spores only
12:30 p.m.	3	Spores only
1:30 p.m.	4	Vegetative growth, some spores
2:30 p.m.	5	Vegetative growth, some spores
3:30 p.m.	6	Vegetative growth, some spores
4:30 p.m.	7	Vegetative growth only
5:30 p.m.	8	Vegetative growth only
6:30 p.m.	9	Vegetative growth marked
7:30 p.m.	10	Vegetative growth marked
8:30 p.m.	11	Vegetative growth marked
9:30 p.m.	12	Vegetative growth abundant
10:30 p.m.	13	Vegetative growth abundant
11:30 p.m.	14	Vegetative growth abundant
12:30 a.m.	15	Vegetative growth very abundant
1:30 a.m.	16	Vegetative growth very abundant
2:30 a.m.	17	Cells show slight internal granulation
3:30 a.m.	18	Cells show marked granulation
4:30 a.m.	19	Granulation advanced
5:30 a.m.	20	Uniting of granules (advanced)
6:30 a.m.	21	Completed spores observed
7:30 a.m.	22	Abundant spores
8:30 a.m.	23	Very abundant spores
9:30 a.m.	24	Very abundant spores

It was thought that possibly the number of spores inoculated into the sterile sand in the preparation of cultures might have some influence upon the length of the spore cycle, as the accumulation of metabolic products is known to affect vegetative growth, and presumably might hasten or retard spore formation. Accordingly, other tests were made using cultures that were prepared by inoculating one set of sand plates with a full-strength

suspension of spores and another with the same diluted to one-tenth of its original strength.

The results of these comparative tests showed no perceptible differences in the progress of spore formation, the spore cycle being completed in both cases in twenty-one hours.

The length of the spore cycle among bacteria is not only of great importance, for the purposes of an investigation like the present; information in regard to this point is also necessary for an adequate application of bacteriological methods to practical problems. It has been the practice of bacteriologists for many years to sterilize certain kinds of culture media by what is known as the fractional sterilization method, which consists in heating the substance at 100°C. or less for a definite length of time on each of three successive days. With the development of home food canning the intermittent process of sterilization has come into wide use, particularly in the Southern States, and the experience of home canners, as well as of bacteriologists in the laboratory, has been that at times this method of treatment fails. An explanation for such failure is to be found in the spore cycle study which indicates that often too long an interval has been allowed to intervene between the first and second heat treatments; types of food destroying bacteria which have short spore cycles find time to return to the spore form again, and thus survive the second, and sometimes the third cooking.

PREPARATION AND STANDARDIZATION OF SPORE SUSPENSIONS

The preparation of uniform spore suspensions was made easy by the loose sand substratum. This, while it furnished optimum conditions for vegetative growth and abundant spore formation, made possible the separation of the filaments and cells so that an even and satisfactory suspension could be obtained in a very few moments by merely shaking a small quantity of the sand culture in a tube of sterile water.

In order to eliminate the possible effects of residual nutrient substances and metabolic products of the culture in the thermal death point tests the spores were washed twice with sterile distilled water, and a final suspension made of the washed spores.

This was accomplished as follows: About 20 cc. of sterile distilled water was transferred aseptically to a sterile 20 by 150 mm. culture tube. By means of a small flat-edged metal scoop, freshly flamed, a quantity of the sand culture was introduced into the tube, the cotton plug returned, and the tube agitated until a sufficient number of spores had been freed into the liquid. The resulting even suspension was then transferred by means of a sterile pipette to a sterile centrifuge tube and centrifuged at high speed to throw down the spores. The supernatant liquid was then pipetted off and more sterile water added to the tube in such a way as to bring the spores into suspension again. Centrifuging was repeated and the clear liquid pipetted away as before. This repeated washing in relatively large quantities of sterile distilled water removed from the spores all substances which might affect their thermal death points in the succeeding tests. The washed spores were then suspended again in sterile distilled water and the suspension standardized by its opacity, following the method described by Brown and Kirwan (1915).

METHOD FOR TESTING THERMAL RESISTANCE OF SPORES

For the accurate determination of thermal death points it is essential that the application of heat be instantaneous, or as nearly so as possible, in order that all may receive identical treatment. Failure in this respect may be responsible for conflicting results or may lead to erroneous conclusions. It is also highly desirable, if not absolutely necessary, that all treated spores be given an opportunity for germination, for the culturing of a small portion, such as a loopful or drop, from a treated suspension of spores is likely to give approximate results only. For these reasons it was not considered best to follow the methods of Bigelow and Esty (1919) which have hitherto been considered especially desirable for work of this sort. The tubes with which these workers made their tests were 7 mm. in internal diameter and 250 mm. long, with walls 1 mm. thick. It takes a perceptible time for heat to pass through 1 mm. of glass and still more for an equilibrium of temperature to be reached in a column of liquid 7 mm. in diameter. It was decided, therefore, that thin walled capillary

tubes should be used to contain the spore suspension to be treated, and that these should be introduced entire into the culture tubes for sterility tests.

It was realized that capillary tubes had the disadvantage of holding but small quantities of the suspension, but the fact that the number of spores in the suspension could be made as large as desired seemed to dispose of this objection.

Owing to the fact that the hydrogen-ion concentration of liquids is altered when heated in soft glass tubes, due to the dissolving out of alkalies, as shown by Esty and Cathcart (1921), "Pyrex," a very hard and insoluble glass was chosen for this work. Tubing having an internal diameter of 4 mm. was drawn out to capillaries of 1 to 1.5 mm. in internal diameter, these cut into lengths of 9 to 10 cm. and the ends sealed immediately in the flame to keep the interior sterile until needed for use.

The spore suspension having been prepared and standardized in the manner described, a quantity was transferred with aseptic precautions to a small sterile shell vial held at a convenient angle by inserting the base in a lump of modeling clay.

The capillary tubes were then charged with the spore suspension according to the following technique: Tubes sufficient in number to meet the requirements of the test were placed in a glass stender dish and covered with alcohol. As each was needed it was picked out with freshly flamed forceps, the alcohol burned off to sterilize the outside, and the sealed tips clipped off by means of a special clipping instrument freshly sterilized in the flame. One end of the tube was then dipped into the spore suspension which rose in the tube by capillary attraction. When sufficient liquid had been taken up the tube was removed, the liquid centered in the tube so as to leave a free space at each end, and the tips of the tube sealed immediately in the flame.

As rapidly as they were filled and sealed the tubes were dropped into a vessel containing cold potassium bichromate-sulphuric acid cleaning solution. This was done to kill any spores adhering to the outside of the tube which had not been destroyed at the time the tips were sealed. They were allowed to remain in this solution until all the tubes were charged. The cleaning solution

was then drained away and the tubes washed in clean cold water. When washed the tubes were transferred to a dish of fresh alcohol kept cold by placing on crushed ice. This precaution was taken to prevent any tendency of the spores to germinate, which might have affected their resistance.

The tests were made in series of fives, that is, five tubes were used in making a resistance test at each time interval.

The source of heat was an electrically heated and controlled constant temperature oil bath, fitted with a motor driven stirring device, which assured a uniform temperature throughout the bath. "Wesson" oil was the heating medium used.

To make the exposure, five of the suspension-charged tubes were removed from the cold alcohol with sterile forceps and transferred to a small aluminum holder, and the receptacle immersed in the hot oil. Both the immersion and the removal were performed as rapidly as possible and the length of exposure timed by the watch.

At the conclusion of the heat exposure the tubes were immediately transferred to a 4-ounce salt-mouth glass stoppered bottle containing fresh acetone, which dissolved off the oil. By means of sterile forceps they were then removed to fresh alcohol to keep them sterile until inoculations into nutrient media could be made. This, in most instances, was done at once, and in no case did the time exceed more than two or three minutes.

The sterility tests were made by flaming the plug and mouth of the culture tube in the usual way and then introducing the capillary tube containing the spore suspension. In preparing the capillary tubes for this inoculation they were withdrawn from the alcohol with sterile forceps and, without flaming, one sealed tip was removed with the freshly flamed clipper. The open end was then inverted over the mouth of the culture tube from which the cotton plug had been removed, and the upper tip of the capillary snipped off. At the same instant the capillary tube was released and dropped into the nutrient broth of the culture tube. The plug was then replaced, and after making certain that the contents of the capillary had been forced up into the medium by the bubble of air formed when the tube touched the medium, the culture was ready for incubation.

These operations, which may perhaps appear complicated from the description, were in reality very simple, and rapidly performed. In all cases the spores exposed to the heat treatment were placed under optimum conditions for germination within five minutes of the time the tubes were withdrawn from the oil bath.

The determination of the thermal death point consisted simply in the incubation of these cultures at 30°C., noting the point at which germination ceased, as indicated by the last positive and the first negative growths in the culture tubes.

It might seem that with so many operations entering into the tests the danger of contamination would be very great and the interpretation of results, therefore, difficult. As a matter of fact, contamination did not occur in 1 per cent of the tests and the results were clear-cut and definite. As was earlier pointed out, contamination when present was easy to detect because of the very unusual and characteristic growth of *Bacillus mycoides* in broth cultures made in this way.

This technique has the merit of assuring uniform and instantaneous exposure of all spores to the heat, and of giving to each the opportunity to germinate.

STUDY OF FACTORS AFFECTING VARIATION IN RESISTANCE OF SPORES

The relation of the hydrogen-ion concentration of the medium to the destruction of bacterial spores has already been given consideration at the hands of Buchanan and his collaborators (1918), Esty and Cathcart (1921), and Bigelow and Cathcart (1921), and it was decided, therefore to eliminate this subject from the present study.

Because so many as yet unknown elements enter into the matter of the relation of food supply, protective colloids, metabolic products, etc., to the resistance of spores it seemed desirable, in so far as possible, to eliminate these factors also from the present investigation.

The influences that might possibly affect the resistance of spores which it was decided to investigate at this time were those of the age of the spores and the environmental factors of temperature

and humidity up to the time of the tests. The plan pursued was as follows:

Sand substratum spore cultures were prepared as previously described, with the modification that sterile clay tops were substituted for the glass covers of the petri dishes. This was done to facilitate the regulation of the humidity of the cultures within the storage chambers. These cultures were incubated at 30°C. for two days to allow for maximum spore formation, as indicated by the spore cycle tests.

At the end of this period of incubation a thermal resistance test upon the young spores, twenty-four to thirty hours old, was performed to establish a point of departure in the determination of thermal resistance variations. The remaining cultures were grouped into series for storage under environmental conditions as follows:

Temperatures—ice-box (approximately 10°), 20° and 30°C. (optimum growth temperature)

Humidities—over a dehydrating agent (CaO), over carefully adjusted sulphuric acid solution (50 per cent humidity), and in water-saturated atmosphere (100 per cent humidity)

The age factor was to be studied by resistance tests made at intervals of thirty days upon each of these series of cultures.

As arranged, series of sand cultures were stored at each of the three temperatures mentioned, in humidity chambers whose atmospheres had moisture contents of approximately zero per cent, 50 per cent, and 100 per cent respectively. Resistance tests performed at thirty-day intervals upon cultures stored under these sets of conditions made possible a study of the influence of age under nine distinct situations, and corresponding studies upon the factors of temperature and humidity.

The results of these tests are presented in tables 1 to 9.

DISCUSSION OF TABLES

Age factor

That age is a factor in determining the resistance of the spores of *Bacillus mycoides* to heat is abundantly shown by the foregoing

results of thermal death time tests. In no case did the resistance of the spores remain constant throughout the period of the tests, or even approximately so. The nearest approach to constancy was observed in the results shown in table 1, but even here perceptible change occurred.

In some instances the degree of resistance increased rapidly during the early days of the test period and then dropped again;

TABLE 1

Showing the results of thermal resistance tests upon the spores of Bacillus mycoides held in a desiccator over calcium oxide in an icebox at a temperature of approximately 10°C., for from thirty to one hundred and eighty days. Exposures in these and succeeding tests made in oil bath maintained at 100°C. Suspension in these and succeeding tests contained 150 million spores per cubic centimeter

LENGTH OF EXPOSURE	AGE OF SPORES						
	24-30 hours	30 days	60 days	90 days	120 days	150 days	180 days
minutes							
½	+++++	+++++	+++++	No test	+++++	+++++	+++++
1	+++−−	+++−−	+++++	+++++	+++++	+++++	+++++
1½	+−−−−	−−−−−	++++0	No test	+++++	++−−0	++−−−
2	−−−−−	−−−−−	++−−−	++−−−	−−−−−	+−−−−	+−−−−
3	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	+−−−−	−−−−−
4	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−
5	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−

Note: Signs used apply similarly to all succeeding tables.

+ indicates positive growth in culture tube following exposure to heat of the spores under test.

− indicates no growth in culture tube.

0 indicates that the test was lost by accident.

It is seen that in a dry, cold environment there was but slight change in resistance of the spores during the entire test period, though there took place a slight increase after the first thirty days of storage.

later to be followed by another increase. The degree of resistance attained in a number of cases was two or three times as great as that of the young spores.

With several series the resistance curve rose abruptly during the first thirty days and then became horizontal, or practically so. Several showed a gradual increase in the resistance of the spores for as long as sixty days.

Temperature factor

Glancing back over the tables it will be noted that in every case, regardless of humidity, and almost without regard to the age of the spores, the greatest resistance was developed at the temperature of 20°C., indicating that a temperature somewhat

TABLE 2

Showing the results of thermal resistance tests upon the spores of Bacillus mycoides held in a dessicator over calcium oxide at a temperature of 20°C., for from thirty to one hundred and eighty days. Exposures made in oil bath maintained at 100°C.

LENGTH OF EXPOSURE	AGE OF SPORES						
	24–30 hours	30 days	60 days	90 days	120 days	150 days	180 days
minutes							
½	+++++	+++++	No test	No test	+++++	+++++	+++++
1	+++−−	+++++	No test	+++++	+++++	+++++	+++++
1½	+−−−−	+++++	No test	No test	+++++	+++++	+++++
2	−−−−−	+++++	+++++	+++++	+++++	+++++	+++++
3	−−−−−	+++++	+++++	++++−	++++−	+++++	+++++
4	−−−−−	++−−−	++++−	−−−−−	−−−−−	++−−−	++++0
5	−−−−−	+−−−−	++++−	−−−−−	−−−−−	+−−−−	+++−0
6	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	+−−−−
7	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−
8	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−

When held in a very dry atmosphere and at a moderate temperature marked changes occurred in the thermal resistance of the spores. Within the first thirty days of storage the thermal death time rose from two to six minutes, indicating a threefold increase in resistance over that of the original spores. This degree of resistance was maintained for another thirty days, and then showed a decided decline. At one hundred fifty days the resistance began to increase again, and at one hundred and eighty days was highest during the period of the tests. The significance of this fluctuation is not known.

below the optimum for vegetative growth, and in most cases well above the minimum, seems to be most favorable for change in spore resistance, at least in the case of *Bacillus mycoides*.

Humidity factor

To evaluate the direct influence of humidity upon change in spore resistance is extremely difficult if not impossible. In com-

TABLE 3

Showing the results of thermal resistance tests upon the spores of Bacillus mycoides held in a dessicator over calcium oxide at a temperature of 30°C., for from thirty to one hundred and eighty days. Exposures made in oil bath maintained at 100°C.

LENGTH OF EXPOSURE	AGE OF SPORES						
	24–30 hours	30 days	60 days	90 days	120 days	150 days	180 days
minutes							
½	+++++	+++++	No test	No test	+++++	+++++	+++++
1	+++−−	+++++	+++++	+++++	+++++	++++0	+++++
1½	+−−−−	+++++	No test	No test	No test	+++++	++−−−
2	−−−−−	+++++	+++++	+++++	+++++	++++−	−−−−−
3	−−−−−	−−−−−	+−−−−	−−−−−	++−−−	−−−−−	−−−−−
4	−−−−−	−−−−−	−−−−−	−−−−−	+−−−0	−−−−−	−−−−−
5	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−

In a dry but warm atmosphere (temperature optimum for vegetative growth) it is seen that a decided increase in resistance to heat developed in the spores during the first thirty days. This increase, while not as great as in those held at 20°C., was never-the-less marked, and was maintained for five months without marked alterations. At the one hundred and eighty-day test the resistance had fallen back to its original position, or essentially so.

TABLE 4

Showing the results of thermal resistance tests upon the spores of Bacillus mycoides held at icebox temperature (approximately 10°C.) in an atmosphere having a 50 per cent humidity, for from thirty to one hundred and eighty days. Exposures made in oil bath maintained at 100°C.

LENGTH OF EXPOSURE	AGE OF SPORES						
	24–30 hours	30 days	60 days	90 days	120 days	150 days	180 days
minutes							
½	+++++	+++++	No test	No test	+++++	+++++	+++++
1	+++−−	+++++	+++++	+++++	+++++	+++++	++++N
1½	+−−−−	+++++	No test	No test	+++++	+++++	+++++
2	−−−−−	+++++	+−−−−	+++++	+++++	+++++	+++++
3	−−−−−	++++−	−−−−−	+++++	+++−−	+++++	+++++
4	−−−−−	−−−−−	−−−−−	−−−−−	++−−−	++++−	−−−−−
5	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	++−−−	−−−−−
6	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−

In a cold atmosphere, half saturated with moisture the spores showed a prompt and marked increase in resistance. In thirty days the degree of resistance had doubled. At the sixty-day period there was an apparent decline, but the results of the succeeding tests indicate that some uncontrolled factor influenced the results of the sixty-day tests. The highest resistance was reached at the time of the one hundred and fifty-day tests.

bination with other factors, such as temperature, it is observed to be very important, as may be seen in the case of the series of spore cultures held at the temperature of the ice-box. It is apparent that the lack of moisture had a direct restraining influence upon the change in resistance of spores held over calcium oxide in the chamber at ice-box temperature as shown in table 1. Here it will be noted that no increase occurred during the first thirty

TABLE 5

Showing the results of thermal resistance tests upon the spores of Bacillus mycoides held at a temperature of 20°C. in an atmosphere having a humidity of 50 per cent, for from thirty to one hundred and eighty days. Exposures made in oil bath maintained at 100°C.

LENGTH OF EXPOSURE	AGE OF SPORES						
	24–30 hours	30 days	60 days	90 days	120 days	150 days	180 days
minutes							
½	+++++	+++++	No test	No test	+++++	+++++	++++0
1	+++−−	+++++	No test	+++++	++++0	++++0	+++++
1½	+−−−−	+++++	No test	No test	+++++	+++++	+++++
2	−−−−−	+++++	+++++	+++++	+++++	+++++	+++++
3	−−−−−	+++++	++++0	+++++	+++++	+++++	+++++
4	−−−−−	++0 0 0	+++−−	+−−−−	++++−	−−−−−	++++−
5	−−−−−	++−−−	−−−−−	−−−−−	−−−−−	−−−−−	++−−−
6	−−−−−	+−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−
7	−−−−−	++−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−
8	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−
9	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−
10	−−−−−	−−−−−	−−−−−	−−−−	−−−−−	−−−−−	−−−−−

Under conditions of medium temperature and humidity the spores showed marked increase in resistance. At thirty days evidences of an especially high resistance are noted in the case of some spores. The high degree of resistance attained for the majority of the spores was retained throughout the period of the tests.

days, although spores held at ice-box temperature but in atmospheres having humidity values of 50 and 100 per cent respectively showed a very marked increase in resistance during this period.

On the other hand, high humidity combined with low temperature resulted in as great changes as those which developed under either high humidity-optimum growth temperature or low humidity-optimum growth temperature conditions.

TABLE 6

Showing the results of thermal resistance tests upon the spores of Bacillus mycoides held at a temperature of 30°C. in an atmosphere having an humidity of 50 per cent, for from thirty to one hundred and eighty days. Exposures made in oil bath maintained at 100°C.

LENGTH OF EXPOSURE	AGE OF SPORES						
	24–30 hours	30 days	60 days	90 days	120 days	150 days	180 days
minutes							
½	+++++	+++++	No test	No test	+++++	+++++	++++0
1	+++−−	+++++	+++++	+++++	+++++	+++++	++++0
1½	+−−−−	+++++	No test	No test	+++++	+++++	+++−−
2	−−−−−	+++++	+++++	+++++	+++++	+++++	+−−−−
3	−−−−−	++++0	+++++	+++++	+−−−−	++++−	−−−−−
4	−−−−−	++−−−	+++−−	+−−−−	−−−−−	++++−	++−−−
5	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−
6	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−
7	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−

In a warm, moderately humid environment the spores, as shown by these results, developed a pronounced increase in resistance within the first thirty days. Some irregularity in the shape of the thermal death time curve is noted, and at the time of the one hundred and eighty-day tests the resistance seems to have decreased considerably.

TABLE 7

Showing the results of thermal resistance tests upon the spores of Bacillus mycoides held at the temperature of the icebox (approximately 10°C.) in an atmosphere having a humidity of 100 per cent, for from thirty to one hundred and fifty days. Exposures made in oil bath maintained at 100°C.

LENGTH OF EXPOSURE	AGE OF SPORES						
	24–30 hours	30 days	60 days	90 days	120 days	150 days	180 days
minutes							
½	+++++	+++++	No test	No test	No test	+++++	
1	+++−−	+++++	+++++	+++++	+++++	+++++	No ma-
1½	+−−−−	++++0	No test	No test	+++0 0	+++++	terial
2	−−−−−	++++−	+++++	+++++	+++++	+++++	for 180-
3	−−−−−	+++−−	+++++	++++−	++−−−	+++++	day
4	−−−−−	+−−−−	++−−−	−−−−−	−−−−−	++−−−	tests
5	−−−−−	−−−−−	+−−−−	−−−−−	−−−−−	−−−−−	
7	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	

In a cold, moisture-saturated atmosphere the resistance of these spores continued to increase steadily for sixty days. After six months of storage the resistance was still more than twice that of spores twenty-four to thirty hours old.

TABLE 8

Showing the results of thermal resistance tests upon the spores of Bacillus mycoides held at a temperature of 20°C. in an atmosphere having an humidity of 100 per cent, for from thirty to one hundred and eighty days. Exposures made in oil bath maintained at 100°C.

LENGTH OF EXPOSURE	AGE OF SPORES						
	24–30 hours	30 days	60 days	90 days	120 days	150 days	180 days
minutes							
½	+++++	++++0	No test	No test	+++++	+++++	+++++
1	+++−−	+++++	+++++	+++++	+++++	+++++	+++++
1½	+−−−−	+++++	No test	No test	+++++	+++++	+++++
2	−−−−−	+++++	+++++	+++++	+++++	+++++	+++++
3	−−−−−	+++++	+++++	+++++	+++++	+++++	+++++
4	−−−−−	++++−	++++0	+++−0	++++−	++++−	+++++
5	−−−−−	−−−−−	++++−	−−−−−	+++++	+−−−0	++−−−
6	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−
7	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−

When held in a moderately warm, moisture-saturated environment the resistance of the spores increased in the same fashion as shown in table 7, but to a slightly greater degree, and the high degree of resistance attained was still retained at the time of the one hundred and eighty-day tests.

TABLE 9

Showing the results of thermal resistance tests upon the spores of Bacillus mycoides held at a temperature of 30°C. in an atmosphere having an humidity of 100 per cent, for from thirty to one hundred and eighty days. Exposures made in oil bath maintained at 100°C.

LENGTH OF EXPOSURE	AGE OF SPORES						
	24–30 hours	30 days	60 days	90 days	120 days	150 days	180 days
minutes							
½	+++++	+++++	No test	No test	+++++	+++++	+++++
1	+++−−	+++++	+++++	+++++	+++++	++++0	+++++
1½	+−−−−	+++++	No test	No test	+++++	+++++	+++−−
2	−−−−−	+++++	+++++	+−−−−	+++++	−−−−−	+−−−−
3	−−−−−	++++−	++−−−	−−−−−	+++++	−−−−−	−−−−−
4	−−−−−	−−−−−	−−−−−	−−−−−	++++−	−−−−−	−−−−−
5	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−
6	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−

With maximum moisture, and optimum growth temperature supplied the spores of this series showed considerable variation in their resistance. The thermal death time curve was quite irregular.

GENERAL DISCUSSION

Having now observed the alteration in thermal resistance of bacterial spores under different conditions as regards age, temperature and humidity, we come to ask ourselves the reasons for these changes.

For many years bacteriologists have held the opinion that spore formation among bacteria represents the response of the organism to an unfavorable environment, and that the spore once formed remains dormant or inactive until a return of conditions favorable for vegetative growth.

That abundant spore formation takes place at temperatures best adapted for vegetative growth, among some species of bacteria at least, has been often observed and has been shown again in the present work with *Bacillus mycoides*. Unfavorable temperature conditions, therefore, do not determine spore formation. Insufficient food supply cannot be the controlling factor because spore formation and vegetative growth proceed side by side. Our own experiments, earlier mentioned, indicate also that the concentration of metabolic products cannot be the determining influence, for spore formation took place as quickly in a dilute suspension of organisms as in one containing ten times as many. Absence of moisture is not responsible for the formation of spores, for every bacteriologist knows that spores form in liquid as well as on solid media.

If, then, neither food supply, moisture, temperature, nor metabolic products which are the factors commonly supposed to affect bacterial activities, control spore formation, may we not look upon this phenomenon of spore formation as one of the normal phases in the ordinary existence of at least certain of the bacteria, which proves of vital importance to the species in that it enables some individuals to survive adverse conditions? May we not look upon the spore, from the standpoint of the bacteria, as a most fortunate provision against adversity rather than as a product of it?

This idea has been furthered by the analysis of the present experimental findings which prove that spores rarely, if ever, are dormant. Increase in resistance under a wide range of conditions

has been observed. This change has been found to take place most rapidly under conditions apparently not the most likely to injure the vitality of the cell but rather under what might be termed "temperate" conditions—conditions suited to positive but rather sluggish vital activities normal to the spore form.

Plant physiologists and plant chemists have come to know that the seed which used to be considered dormant is in reality undergoing fundamental internal alterations even though the change cannot be detected from its external appearance. In like manner also, tubers and other storage organs of plants which during the winter period have been supposed to be in a dormant condition have been found to have undergone great changes internally.

We are coming, therefore, to a realization that strict dormancy among living forms rarely, if ever, exists and that what has been interpreted as such is merely a retardation in the rate of vital activities.

In the light of evidence at hand it seems reasonable to conclude that variations in resistance of bacterial spores under differing environmental conditions are the results of normal spore transformations which are retarded by some and possibly accelerated by other conditions of environment.

The results of the present investigation are of general scientific interest and of practical significance.

That virulence among pathogenic bacteria may be altered in either direction by modifications in cultural practices has long been known. That virus once formed may be weakened by certain storage treatments, as is done in the preparation of the attenuated virus of rabies for the Pasteur treatment, has likewise long been known and put to practical use in the practice of human and of veterinary medicine. It has been learned also that under certain conditions of cultivation a spore-forming organism may be prevented from forming spores normally, or if doing so, may be made to form spores possessing different degrees of resistance.

The assumption has been that once a spore has been formed its resistance is a fixed property. That this idea must be abandoned seems evident, for what has hitherto been looked upon as a constant has been shown to be a variable.

The results of the present study are of practical significance in two fields: First, that which is concerned with disinfection and disinfectants; and the second, that which involves food preservation.

A few only of the disease-producing bacteria are of spore forming types, but those which possess this property are extremely dangerous. It is of importance, therefore, that all methods of disinfection and all disinfectants be selected and applied in such a way as to take into account not only the variations in the resistance of different strains of these organisms but also the changes in resistance almost certainly possible in all spores.

Of particular importance is this matter as it applies to the testing and standardization of chemical disinfectants. Germicidal values are determined, in many instances at least, by the ability of the substance in different dilutions to destroy the spores of *Bacillus anthracis* or other pathogenic spore-forming bacteria. It is absolutely essential for safety that the resistance of the spores used in these tests be as uniform as possible, and the alterations in resistance occurring under storage as well as under cultural conditions must be taken into account.

The significance of these findings as they concern food preservation lies in the importance which thermal death points of bacterial spores have in determining the temperatures and time periods of the processing, or cooking of the food in the can.

The canning industry is rapidly getting away from rule-of-thumb practices and more and more is basing its operations upon the findings of research laboratory workers. The rate of heat penetration into the cans of food during the processing has been extensively studied; the relations of the character of the food and the character of the pack to ease of sterilization have been investigated; bacteria causing food spoilage have been isolated and subjected to careful observation; and the thermal death points of numerous highly resistant spore-forming types have been determined. Upon the basis of these findings new processing schedules have been developed and the results of careful laboratory experiments are being applied in the industry.

This has been done under the assumption that the thermal

death points observed for particular organisms represented fixed values. From the present findings we are led to conclude that these values are not constant, and unless a considerable margin of safety is provided for in the standardization of food processing schedules the new practices, at times, may be expected to fail. In determining the thermal death points that are to serve as the basis of new processing schedules bacteriologists must be as certain as possible that the resistance shown by the test spores represents the highest degree attainable by them.

SUMMARY

The object of the present investigation was to throw light upon the important subject of thermal resistance of bacterial spores, with special reference to changes in resistance which have been observed to occur, and which it is thought may have an important bearing upon the problem of food preservation.

The organism selected for the study was the *Bacillus mycoides* which, because of its cultural and physiological characteristics, is particularly well adapted to the work.

The experiments were confined primarily to a determination of the influence of age and of the environmental factors of temperature and humidity upon variations in thermal resistance of spores, and data were obtained at thirty-day intervals upon spores stored under nine different sets of environmental conditions. A study of the spore cycle of the organism under test; the development of special cultural methods in order to provide for maximum spore formation and the preparation of satisfactory spore suspensions; and the development of a more satisfactory technique for making the resistance tests, were necessary parts of the investigation.

The results of the tests may be summarized briefly as follows:

1. In a cold, dry environment there was practically no change in the resistance of the spores during the first thirty days of storage, but a small increase was manifest by the time of the sixty-day tests which was maintained for the remainder of the test period.

2. When held in a very dry atmosphere and at a moderate temperature marked changes occurred. Within the first thirty days of storage the thermal death time rose from two to six minutes, indicating a threefold increase in resistance over that of the original spores. This degree of resistance was maintained for another thirty days, and then showed a decided decline. At one hundred and fifty days the resistance began to increase again, and at one hundred and eighty days was at a maximum point. The significance of this fluctuation is not known.

3. In a dry but warm atmosphere (temperature optimum for vegetative growth) a decided increase in resistance to heat developed in the spores during the first thirty days. This increase, while not as great as in those held at 20°C., was marked, and was maintained for five months without material alterations. At the time of the one hundred and eighty-day tests the resistance had fallen back to nearly its original position.

4. In a cold atmosphere, half saturated with moisture the spores developed a prompt and marked increase in resistance, the degree of resistance doubling within the first thirty days. The highest point in this series was reached at the time of the one hundred and fifty-day tests.

5. A pronounced increase in resistance resulted when the spores were held under conditions of medium temperature and humidity. An especially high resistance was shown by some of the spores at the time of the thirty-day tests and a considerably increased resistance for the majority of the spores was not only attained but also retained throughout the period of the tests.

6. In a warm, moderately humid environment the spores developed a marked increase in resistance within the first thirty days. The death time curve for the entire period of the tests, however, was somewhat irregular.

7. In a cold, moisture-saturated atmosphere the resistance of the spores continued to increase steadily for sixty days, and after six months of storage was still more than twice that of spores twenty-four to thirty hours old.

8. When held in a moderately warm, moisture-saturated environment the resistance of the spores increased in the same

fashion as the preceding but was slightly less pronounced. The high degree attained, however, was still retained at the time of the one hundred and eighty-day tests.

9. With maximum moisture and optimum growth temperature supplied considerable variation in resistance was noted. The thermal death time curve was quite irregular.

CONCLUSIONS

A careful analysis of the experimental data presented leads to the following conclusions:

1. The bacterial spore is not dormant under ordinary conditions, as has commonly been supposed, but is instead sluggishly active.

2. The resistance of spores to heat is not a fixed property but a variable one, the degree of resistance being influenced by age, the temperature and humidity of the environment, and possibly other factors.

3. The highest resistance to heat develops under conditions of moderate temperature and humidity, and is probably reached by the time the spores are sixty days old. Spores of different species of bacteria may be expected to vary somewhat in this respect.

4. Change in resistance takes place most slowly when spores are dry and cold, but low temperature accompanied by high humidity results in the development of a high degree of resistance.

5. In determining the thermal death points of spores that are to serve as the basis of processing schedules for canned foods the bacteriologist must take into account the change in resistance of spores under various conditions, and be as certain as possible that the resistance shown by the test spores represents the highest degree attainable by them.

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APPENDIX

The following is a condensed résumé of the literature bearing upon the subject of bacterial spore resistance:

1888 Globig (1888) found that spores of the "potato bacillus" withstood exposure in streaming steam at 100°C. for 5 to 6½ hours; and in superheated steam at 113 to 116°C. for 25 minutes; at 112 to 123°C. for 10 minutes; at 126°C. for 3 minutes; and at 127°C. for 2 minutes. Death instantaneous at 130°C.

1889 Esmarch (1889) found that resistance of spores of *B. anthracis* was affected by nutrient medium used.

Geppert (1889) reported variation in resistance of spores of *B. anthracis* even when grown under identical cultural conditions.

Fraenkel (1889) confirmed the findings of Geppert (1889) and concluded that variation in resistance is an inherent property of different strains and not affected by conditions of environment.

1895 Miquel and Zattraye (1895) found that moist spores of *B. subtilis* in cultures 3 to 4 weeks old survived exposure for 15 minutes at 104.4 to 108.3°C.; 30 minutes at 102.8 to 106.3°C.; 1 hour at 102.9 to 104.7°C.; and 4 hours at 99.7 to 100.1°C.

Christen (1895) described organisms whose spores resisted 100°C. for periods of 4 to 16 hours; 105°C. for 1 to 4 hours; 110°C. for 1 to 2 hours; 115 to 116°C. for ½ to 2 hours; and 130°C. for 5 minutes.

1896 Cambier (1896) found that spores of soil organisms exposed to dry heat survived after 3½ hours treatment at 110.7°C., but were killed in 1½ hours at 124°C.; and in 15 minutes at 138°C. In air-dry soil the spore survived 111°C. for 4½ hours; 136.9°C. for 3 hours; 156.5°C. for 2 hours; and 180°C. for 35 minutes. All were destroyed in 5 minutes at 200°C.

1897 Kronig and Paul (1897) confirmed the findings of Geppert (1889).

Van Ermengem (1897) reported that the spores of *B. botulinus* were only slightly resistant to heat, being destroyed in 1 hour at 80°C.

1899 Weil (1899) confirmed the work of Esmarch (1889) and noted that temperature of incubation appeared to affect resistance of spores.

1902 von Wahl (1902) found 2 hours insufficient time to sterilize carrots, asparagus, and peas packed in glass containers when exposed to streaming steam at 100°C. Spores of an organism in carrots survived 3½ hours processing in water at 100°C.

1904 Neide (1904) described 3 organisms the thermal death points of whose spores varied from 15 to 38 minutes at 100°C.

1905 Blau (1905) reported the thermal death times for spores of various bacteria exposed to temperature of boiling water as follows: *B. subtilis*, 175–180 minutes; *B. robustus*, 450 to 480 minutes; *B. calidus*, 450 to 480 minutes; *B. cylindricus*, 1140 to 1200 minutes; and *B. tostus*, 1140 to 1200 minutes.

1908 von Hibler (1908) secured constancy in the resistance of spores of *B. anthracis* by using cultures that were "neither too old nor too young." Acidity and long storage in incubator found to affect resistance of spores.

1915 Shanly (1915) studied heat resistance of spores of 23 different species. The least resistant, *B. cereus*, survived exposure to 75°C. for 1 hour but was

killed was 80°C. for same period. Others, identified only by numbers, survived after 1 hour at 100°C. Variation in resistance of different strains of same species noted.

1918 Buchanan, Thompson, Orr and Bruett (1918) gave particular attention to effect of preliminary canning operations upon thermal death points. They also determined the relation of the hydrogen-ion concentration of the product to heat resistance of bacteria and their spores.

1919 Bruett (1919) found that scalding followed by chilling is not an effective means of spore destruction.

Burke (1919) reported that while exposure to a temperature of 100°C. may not kill spores of *B. botulinus* their vitality is weakened so that germination is delayed. Spores of this organism survived 3½ hours boiling at 100°C., and 5 hours boiling was considered insufficient to sterilize. Fractional sterilization, because of delayed germination, was held of doubtful value.

Normington (1919) in studies of organisms isolated from cold-packed canned peas found that all withstood 10 to 15 pounds steam pressure in the autoclave for 10 to 20 minutes.

Bigelow and Esty (1919) developed improved method for determining spore resistance. Spores of one strain destroyed only after 16 hours boiling at 100°C.; 100 minutes at 110°C.; 50 minutes at 115°C.; 10 minutes at 120°C.; and 4 minutes at 125°C. Initial number of spores and H-ion concentration of medium were found to affect the time required to sterilize.

Esty and Williams (1919) reported resistance tests upon organisms isolated from canned foods. Considerable variation was noted in resistance, ranging from 1½ to 17 hours at 100°C. True thermophiles were found most resistant.

Thom, Edmondson and Giltner (1919) found spores of *B. botulinus* from canned asparagus survived steaming at 116°C. for 15 minutes, and 100°C. for 1 hour.

Dickson, Burke and Ward (1919) reported upon 8 strains of *B. botulinus*. All survived 3 hours heating at 90°C.; 7 of them 3 hours at 95°C.; and 6 of them 2 hours at 100°C. Addition of 5 per cent of lemon juice to meduim did not prevent growth or formation of toxin but lowered the death point of spores. Spores treated in presence of animal and vegetable protein.

1920 Bigelow and Esty (1920) studied relationship of temperature and time to spore destruction in thermophiles from spoiled canned foods. Destruction of spores in one culture as follows: 1320 minutes in boiling water at 100°C.; 690 minutes at 105°; 225 minutes at 110°; 84 minutes at 115°; 23 minutes at 120°; 8 minutes at 125°; 3.5 minutes at 130°; 1.5 minutes at 135°; and 1 minute at 140°C. Nineteen thermophiles studied showed same time-temperature relationships.

Fenger, Cram and Rudnick (1920) found thermal death points of five organisms isolated from ligatures and sutures, when heated in non-aqueous liquids, to lie between 150 and 160°C. for 1 hour.

Donk (1920) found that spores of a thermophile isolated from canned corn required 17 hours to kill when heated in corn broth at 100°C. with 12,500

spores per 1 cc.; and 11 minutes when heated at 120°C. with 50,000 spores per 1 cc.

1921 Weiss (1921) found that free spores of *B. botulinus* were killed within five hours at 100°C.; 40 minutes at 105°C.; and 6 minutes at 120°C. Young moist spores, more resistant than old, and spores 1 month old found three times as resistant as those 5 months old. More resistant individuals changed more rapidly than less resistant ones. Thermal resistance of emulsions of young spores increased as concentration of emulsion increased. Sodium chloride lowered thermal resistance. Increase in H-ion concentration on acid side of neutrality, and of hydroxyl-ions on alkaline side lowered resistance of spores. Work on spores of *B. botulinus*. In another paper same author (1921) showed relation of H-ion concentration, consistency of food material, concentration of sirup present, etc., to thermal death point of *B. botulinus;* acid foods requiring less severe treatment to sterilize than non-acid substances.

Bigelow (1921) gave the thermal death times of 15 typical thermophiles when exposed to different temperatures as follows: At 100°C., between 788 and 834 minutes; at 105°, between 383 and 405 minutes; at 110°, between 117 and 122 minutes; at 115°, between 40 and 44 minutes; at 120°, between 11 and 12 minutes; at 125°, between 3.9 and 4.6 minutes; at 130°, between 1.7 and 2.2 minutes; at 135°, between 0.7 and 0.9 minutes; and at 140°C., between 0.6 and 0.9 minutes.

1922 Dickson and his associates (1922) noted marked variations in resistance of spores of *B. botulinus*. Tests on 40 strains made at 100°C. showed survival period varying from thirty minutes to six hours. About 95 per cent spores were destroyed in short time, some of remainder highly resistant. Spores from old cultures more resistant than those from young, and resistance greater in neutral media than in acid of alkaline media. Noted delay in germination, in one case for 330 days.

Esty and Meyer (1922) found spores of *B. botulinus* more resistant than practically any other anaerobe, requiring 4 minutes at 120°C. or 330 minutes at 100°C. to kill. Spores in juices of 17 different kinds of canned foods showed variation in resistance of from less than 10 minutes to 230 minutes at 100°C.

Tanner and Dack (1922) studied resistance of spores of *B. botulinus* to dry heat. Spores survived 110°C. after 2 hours; 140°C. from 15 to 60 minutes; and 160 to 180°C. from 5 to 15 minutes.

1923 Esty (1923) found resistance of 112 strains of *B. botulinus* to vary from 3 to 75 minutes at 105°C. Maximum resistance given as 330 minutes at 100°C.; 110 minutes at 105°C.; 33 minutes at 110°C.; 11 minutes at 115°C. and 4 minutes at 120°C. Greater resistance shown in heavy suspensions than in light.

Wyant and Tweed (1923) found that for 8 organisms isolated from "flat-sour" canned peas sterilization was accomplished at above 80°C. and below 110°C. after 10 minutes heating.

Tanner and McCrea (1923) reported resistance of spores of *B. botulinus* sealed in tubes exhausted to 17 mm. Spores destroyed within 5 hours at 100°C.; 2 hours at 105°C.; 1½ hours at 110°C.; 40 minutes at 115°C.

and 10 minutes at 120°C. Longer time required to destroy spores of same age in open tubes than in tubes exhausted and sealed.

1924 Esty (1924) made inoculation experiments upon peas and corn using suspensions containing different numbers of spores. Showed that spoilage in canned product was materially increased when number of spores was increased, due to especially resistant individual spores in suspensions.

OBSERVATIONS ON "PIN POINT COLONY" ORGANISMS IN THE BALTIMORE MILK SUPPLY

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INTRODUCTION AND LITERATURE

It seems to be quite generally agreed that the "pin point colonies" which occasionally appear on plain agar plates inoculated with diluted milk for the purpose of estimating the number of organisms present may be due to a variety of causes.

Yates (1923) suggested that since the trouble was first recognized, coincident with the change in the method of adjusting the reaction of culture media and the more or less general use of chlorine solutions in dairies and on farms, there might possibly be a connection between one or both of these procedures and "pin point colonies."

Ayers and Johnson (1924) found the appearance of these colonies from pasteurized milk from one milk plant to be due to a thermophilic organism which they named *Lactobacillus thermophyles*. They also suggested that crowded plates, the reaction of the medium and heat resistant non-thermophilic bacteria might be possible causes.

Cooledge (1924) by a special technique isolated alkali producing thermophilic bacteria from milk and found that these organisms when present in great numbers overcame the unfavorable reaction of the medium and developed as typical pin point colonies.

Harding and his collaborators (1924) have demonstrated that there are present in all classes of milk organisms which will grow at the pasteurizing temperature. We are not sure whether these organisms produced pin point colonies or not.

OBSERVATIONAL AND EXPERIMENTAL

For a little over two years we have observed in connection with our routine milk control counts that we believe to be typical cases of "pin point colonies." What we call typical cases are not due to crowded plates, for counts made from low dilutions (in so far as they can be counted at all because of their extreme minuteness) are much higher than those from higher dilutions. This would not be true if the "pin point colonies" were due to crowding. They are not due to differences in the reaction of the culture medium in so far as these differences exist prior to incubation. However, in this connection it must be borne in mind that the reaction of the medium in the plates after incubation is rarely the same as it was before incubation. Due to the activity of the developing organisms it is practically always changed. Usually the low dilutions become more alkaline than the higher dilutions, although there may be a change in the acid direction.

Late in the year 1922 we became convinced of the typical appearance of these plates. In order to report our findings as accurately as possible we estimated the number of colonies as best we could and reported the counts with the notation "pin point colonies." This signified that there were at least as many organisms present as reported and probably a great many more. This procedure has been in operation for a little over two years.

It has been our general observation that these cases of "typical pin point colonies" were much more numerous in the winter. In order to establish our observation in this regard we went over our records and calculated the percentage of the total number of examinations made each month which had shown "typical pin point colonies." This was done for each of the three main classes of milk which we analyze. Figure 1 shows the curves constructed ed from these figures for the last two years.

In order to determine if possible the organism or organisms producing these colonies we collected cultures from typical plates over a period of two months (December 15, 1923 to February 15, 1924). One or two colonies from each plate were fished under the microscope and inoculated into litmus milk. These cultures

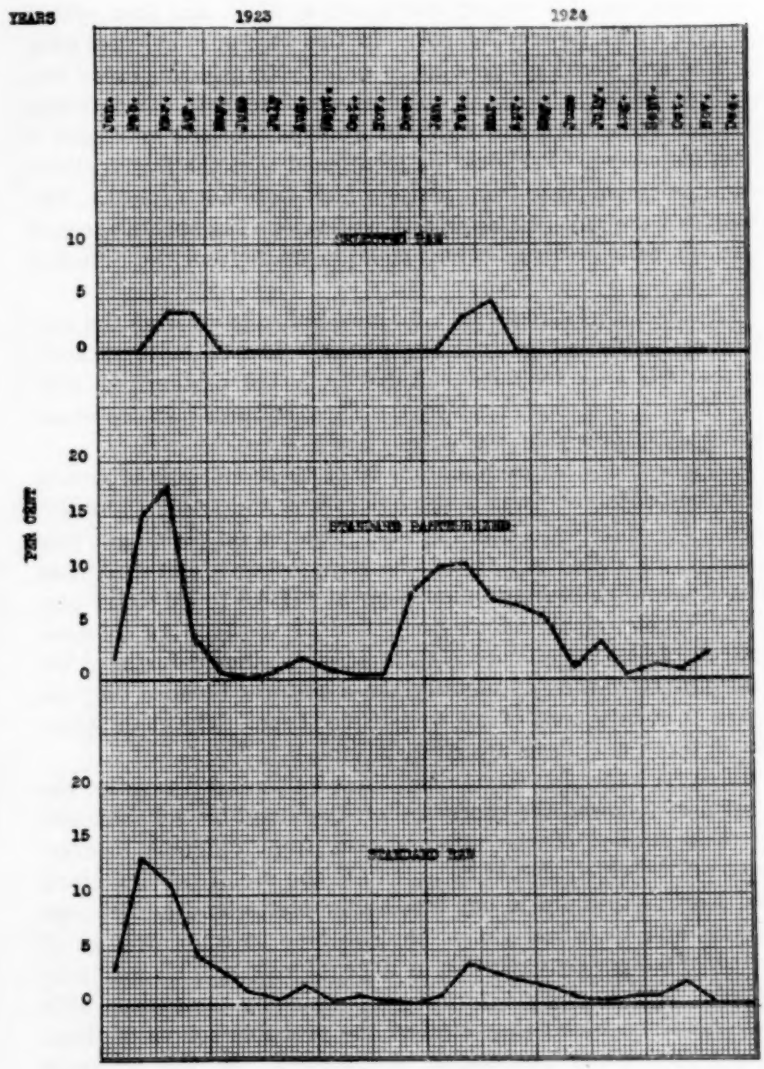

FIG. 1

were incubated for twenty-four hours at 37°C. and then plated on Ayer's milk powder Agar A. In this way pure cultures were secured and were kept growing in milk by transferring every two or three weeks. In all, 52 cultures were secured. Twenty-one of these came from raw milk, 26 from pasteurized milk, and 5 from ice cream. It should be mentioned that the five cultures from ice cream were from plates which were not typical. The colonies were quite small, but they developed in such a manner that approximate check counts could be made from the different dilutions.

The 21 cultures from raw milk included cultures from the raw milk of 5 different pasteurizing plants. The 26 cultures from pasteurized milk included cultures from the pasteurized milk of 7 different pasteurizing plants. The 5 cultures from ice cream included cultures from one plant only.

On morphological examination 50 of these cultures proved to be streptococci, one was a staphylococcus and one a spore former. These last two cultures when replated on plain agar produced large colonies. All of the streptococcus cultures when plated in pure culture on plain agar at pH 6.8 to 7.0 either produced typical small colonies or failed to show up as colonies. Sixteen of the streptococcus cultures were studied further. We have not attempted to identify these cultures because they do not seem to be typical of any well defined type. The characteristics of these organisms are given in table 1.

In so far as we have examined these cultures we have for the most part followed the procedure of Ayers and his collaborators in their studies of the streptococcus from different sources. (Exceptions which should be noted are the use of rabbits' blood and North's medium in our blood agar plates, and of National Aniline and Chemical Co. methylene blue in concentration of 0.1 cc. of 0.5 per cent solution in 10 cc. of milk.) This was done in order that we might determine if possible by comparison with the organisms which they studied the probable source of these "pin point colony" producing streptococci. In this we were disappointed since our cultures do not seem to be typical of any class which they studied, with the possible exception of the 7

TABLE 1

Characteristics of "pin point colony" streptococci

NUMBER OF CULTURES	SOURCE	HEMOLYSIS	CHAINS	METHYLENE BLUE 37°C.	LITMUS MILK	FERMENTATION*							ACID PRODUCTION IN LITMUS MILK†			COLONIES ON PLAIN AGAR WHEN IN PURE CULTURE
						Glucose	Lactose	Sucrose	Salicin	Mannitol	Raffinose	Inulin	20°C.	37°C.	50°C.	
2	Ice cream	Alpha green	Short	Complete reduction 18 hours, coagulated 24 hours	Acid coagulated	+	+	+	+	+	+	−	++	+++	−	+
1	Pasteurized milk	Alpha green	Long	Complete reduction 18 hours, coagulated 24 hours	Acid coagulated	+	+	+	−	−	−	−	−	++	−	−
2	Pasteurized milk	Alpha green	One long; one short	Partial reduction 48 hours, coagulated 72 hours	Acid coagulated	+	+	+	−	−	−	−	−	++	−	-
3	Pasteurized milk	Gamma green	Long		Acid coagulated slowly	+	+	+	−	−	−	−	−	+	−	-
1	Pasteurized milk	Alpha green	Short		Acid	±	±	−	−	−	−	−	±	+	−	-
7	Raw milk	Alpha green	Long		Weak acid	−	−	−	−	−	−	−	±	+	−	+

* Culture tested with methyl red. + indicates final acid color pH 4.6 or lower. ± indicates neutral range approximately pH 5.4 to 4.8. − indicates that cultures did not develop enough acid to appreciably change the color of methyl red pH 5.8 or higher.

† − indicates no change in the color of litmus after five days' incubation. ± indicates the first slight change in three to five days. + indicates a definite change in three days. ++ indicates a definite change in two days. +++ indicates a definite change in one day.

cultures from raw milk, which show some similarity to their cultures of *Streptococcus acidominimus*, which they found was one of the minor udder types.

In order to approximate the optimum growing temperature for these organisms, three sets of litmus milk tubes were inoculated in the same manner. One set was incubated at 20°C., one set at 37°C. and one set at 50°C. The rapidity with which acid was produced as determined by a change in the color of litmus, was taken as indicative of the comparative rate of growth at the different temperatures. The results of these tests are included in the table.

In the last column of the table is indicated the ability of these organisms to produce colonies on plain agar (pH 6.8 to 7.0) when inoculated in pure cultures and without the addition of milk.

DISCUSSION

It would appear from the data presented above that what we have considered "typical pin point colonies" have a fairly definite seasonal occurrence. In each of two years we have found them to occur with the greatest frequency in the months of January, February, March, and April. They occurred at the same time in both raw and pasteurized milk. This fact would indicate that the "pin point colony trouble" which we have been observing has its origin at the farm. However, in examining the organisms responsible for these colonies we found several varieties of streptococci, and those from pasteurized milk were different than those from raw milk. Our cultures were not collected with the idea of determining the predominant types in raw milk and pasteurized milk. To establish a difference in type would require the study of a great many more cultures. Other points to consider are the dilutions of the milk in the original plates and the flora (other than "pin point" colony organisms) present in the milk. What part other organisms may play in making conditions suitable for the growth of a particular organism when they are associated on the same plate is a subject we know little about.

It should be noted that these organisms occur in the milk

supply of Baltimore coincident with the spring freshening season for cows on the Baltimore milk shed. Whether streptococci are more numerous in the udder of cows or about the farm at this time of the year we do not know. However, on purely theoretical grounds, udder trouble would be expected to be quite prevalent at this time of the year.

We do not propose to draw any conclusions from this work as to the connection between these organisms and mastitis in cattle. Much more work would be necessary to determine such a fact. However, it may be said that should such a relationship be established, the occurrence of "typical pin point colonies" when due to streptococci might prove to be a valuable index by means of which udder trouble could be located; just as "pin point colonies" when due to thermophiles have proven of value in locating trouble in pasteurizing plants.

SUMMARY

The literature indicates that "pin point colonies" on milk plates may be due to a variety of causes, such as crowded plates, reaction of medium, thermophilic organisms, and streptococci.

We have found "pin point colonies" to occur on our routine plates for the control of milk with the greatest frequency in the months of January, February, March and April.

The organisms responsible for the colonies were found to be streptococci.

The possible relationship of these organisms to udder trouble and the significance which could be attached to "pin point colonies" when due to streptococci should it be shown that these organisms indicate mastitis have been discussed.

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THE OXALIC ACID TEST FOR INDOL

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The oxalic acid test for indol is one which has received little attention in spite of certain features which would seem to render it of considerable value. It was apparently first applied to bacteriological work by Pittaluga (1908) and Morelli (1909), but failed to gain general recognition and only a few other references to it are to be found. Recently Holman and Gonzales (1923) have again brought forward the method and believe that it is entitled to more widespread use.

The test is based upon the formation of a pink color upon contact, under proper conditions, of volatile indol and dry oxalic acid crystals. Absorbent paper or filter paper is dipped in an aqueous solution of oxalic acid, dried, cut into strips and a strip of the paper suspended from the cotton stopper in the mouth of the tube containing the culture to be tested. As much of the surface of the paper as possible should be exposed inside the tube, care being taken that it does not come in contact with the culture. If indol is formed it volatilizes at incubator temperature, or even at room temperature, and the oxalic acid paper becomes pink. In the absence of indol the paper remains white. Holman and Gonzales report obtaining positive tests on white tape or on absorbent cotton plugs which were dipped in oxalic acid solution and dried.

Although the advantages of such a test have been emphasized before, nevertheless it may be permissible to restate them here, since many are not familiar with the method. In the first place, the color indicative of a positive test is obtained on the paper or cotton plug in the top of the tube, thus automatically eliminating non-volatile compounds related to indol which might give

confusing color reactions when the test reagents are added directly to the medium. The culture is not destroyed by performing the test so that the production of indol, or at least its volatilization from the medium, can be followed through a considerable period on the same culture, inserting new oxalic acid papers after the first have become pink. Also, the test can be applied to cultures grown on solid media such as agar slants or to those grown in very turbid media or in infusions of chopped meat where the usual tests could not be performed. In view of these advantages it would seem that this method is deserving of more attention than it has received in the past. Our purpose has been to review it with especial reference to its delicacy and accuracy as compared with the more commonly used tests.

The factors controlling development of the pink color upon contact of indol and oxalic acid are apparently not entirely understood. The color does not appear if the oxalic acid paper is wet, nor does it appear upon mixture of solutions of indol and oxalic acid. We have found that filter paper strips dipped in a mixture of indol and oxalic acid solutions of varying concentrations remained colorless until the papers had dried, whereupon the pink color appeared. Holman and Gonzales (1923) believe it essential that the crystals of oxalic acid must be very small or distributed on some finely divided material, otherwise the pink color does not appear. They report that there was no color change when indol was allowed to volatilize through packed oxalic acid crystals.

Before comparing this method with other indol tests it seemed desirable to conduct a few preliminary experiments with regard to preparation of the oxalic acid papers. Attention was given especially to the strength of the oxalic acid solution and to the type of paper used. Solutions containing respectively 2, 4, 6, 8, 10 and 12 grams of oxalic acid, $(COOH)_2 \cdot 2H_2O$, per 100 cc. of distilled water were prepared and filter papers immersed in each of them. The papers were then air dried, cut into strips and used for comparative tests with sterile broth in tubes to which small amounts of a weak solution of indol had been added. In these tests the papers prepared from the two weakest solutions

exhibited a lighter shade of pink, especially with small amounts of indol, and seemed to be less desirable. Little if any difference could be detected between the others. In a later experiment a hot saturated solution of oxalic acid was used for impregnating the filter paper. The results secured here appeared to offer no advantage in depth of color. Several different brands of filter paper, both hard and soft, and a special bibulous paper were used with approximately the same results. Absorbent cotton plugs moistened in the oxalic acid solution and dried were also used. It seemed easier, however, to prepare the papers beforehand and to use them as needed rather than to treat each cotton plug separately, and so throughout our work we have used filter papers or bibulous paper impregnated in an 8 to 10 per cent solution of oxalic acid.

The effect of the reaction of the medium upon the volatility of indol would seem to be important since it might affect the delicacy of the test. Zoller (1920) has reported the effect of reaction upon the volatilization of indol from a medium containing 2 per cent Difco pepton, 0.1 per cent glucose and 1 per cent dibasic potassium phosphate adjusted to varying hydrogen ion concentrations. Distillation was accomplished over the free flame of a Bunsen burner. At pH 5.0, 81 per cent of the indol distilled in the first 75 cc.; at pH 7.0, 92.5 per cent; at pH 8.0 to 10.5, 99 to 100 per cent of the indol appeared. In another experiment, air bubbled for one hour through an aqueous indol solution held at 50°C. removed slightly less than 20 per cent of the indol at pH 5.0, about 31 per cent at pH 6.0, 38 per cent at pH 7.0 and 45 to 48 per cent at pH 8.0 to pH 10.0. While the greatest lost in each case occurred in the range of pH 8.0 to 10.5. it is nevertheless apparent that a fairly large proportion escaped even at the more acid reactions. Whether sufficient indol to give a positive test with oxalic acid paper would escape from an acid medium at incubator temperature remained to be determined.

A sample of plain nutrient broth (1 per cent Difco pepton and 0.3 per cent meat extract) was divided into four separate lots and each was adjusted to a different degree of acidity or alkalinity.

Varying quantities of a dilute indol solution were then added to each of the different lots to give concentrations ranging from 0.001 to 0.2 mgm. indol per cubic centimeter of broth. The broth was tubed in 5 cc. quantities and oxalic acid papers inserted in the tubes. This experiment was repeated several times with samples ranging from pH 4.8 to 9.2. In each case a distinct positive test was obtained within twenty-four hours in the tubes containing the greatest concentrations of indol, irrespective of reaction. Where only small quantities of indol were present the effect of the reaction was questionable. In one experiment with 0.005 mgm. indol per cubic centimeter of broth, a very faint test was obtained after forty-eight hours in two tubes, at pH 8.0 and 9.2 respectively; two other tubes, at 4.8 and 6.8, gave questionable or negative results. In several other experiments the distinction was not as clear cut and very little if any difference was apparent between acid and alkaline media. However, in a general way it may be stated that the test appeared to be reliable at different reactions in all cases except those where only small amounts of indol were present. These cases gave conflicting results. Evidently there is at least a possibility that cultures forming only small quantities of indol or those grown in a medium of low tryptophane content, especially if acid or neutral in reaction, may be erroneously recorded as indol negative.

A comparison of the delicacy of the oxalic acid test with several of the commonly used indol tests was next made. For this purpose the following were employed: The Ehrlich test with paradimethylamidobenzaldehyde, the Salkowski test with sodium nitrite and sulphuric acid, the vanillin test and of the Goré test. The technic used in applying the first three of these tests followed that recommended in the Manual of Methods by the Committee on Bacteriological Technic. In each case the reagents are added directly to the culture. The Goré test (1921) has recently been described and is essentially a modification of the Ehrlich test. The reagent with the addition of potassium persulphate is applied to the cotton stopper instead of being added to the medium, the culture is heated to drive off indol and the typical color of the positive test appears on the cotton.

These tests were compared by applying them to dilute solutions of indol in broth or to cultures. A 0.1 per cent solution of indol was made in 95 per cent ethyl alcohol and from this further dilutions were made in sterile broth to give a series of tubes with concentrations of indol ranging from 0.1 to 0.0001 mgm. per cubic centimeter of broth. The different lots of broths with varying concentrations of indol were then filled into test tubes in 5 cc. amounts and each of the tests applied to a series of tubes. The results of the oxalic acid test were recorded after incubation at 37°C. In another experiment a broth culture of *Bact. aerogenes* (indol negative) was used for dilution of the indol instead of the sterile medium. A forty-eight-hour culture, exhibiting luxuriant growth and heavy turbidity, was killed by steaming in the Arnold and varying quantities of indol were then added to the killed culture and the tests applied as before. In this case, of course, the colors obtained in those tests where the reagents are added directly to the medium must be observed through the turbid growth and the conditions more nearly simulate those under which the tests are usually applied in bacteriological work.

The result of these experiments, shown in tables 1 and 2, are essentially similar in that the Ehrlich, Vanillin and Goré tests are more sensitive than the oxalic acid paper. When the tests are applied to clear broth (table 1) the Ehrlich and vanillin tests were capable of detecting indol in quantities as small as 0.001 mgm. per cubic centimeter of broth, i.e., 1 part per million. However, when applied to turbid cultures the highest dilution to give a faintly positive test was that containing 0.002 mgm. indol per cubic centimeter, or 1 part in 500,000. Also, under these conditions the Goré test was as sensitive as the others. The oxalic acid papers are apparently less delicate, since a positive test was never obtained with less than 0.005 mgm. indol per cubic centimeter, or 1 part in 200,000. It should be pointed out in this connection that the reaction of the broth as well as of the killed culture to which these tests were applied was pH 6.8. In our hands the Salkowski test has given the least satisfactory results of any of the tests studied.

Following our work with indol added to sterile broth or killed cultures, we next used cultures grown in several different media:

TABLE 1

Results of the various tests applied to known dilutions of indol in sterile broth

		DILUTIONS OF INDOL PER CUBIC CENTIMETER OF BROTH							CONTROL
		0.1 mgm.	0.01 mgm.	0.005 mgm.	0.002 mgm.	0.001 mgm.	0.0005 mgm.	0.0002 mgm.	
Oxalic acid paper†	24 hours	+	?	−	−	−	−	−	−
	48 hours	++	+	?	−	−	−	−	−
	96 hours	+++	+	+	−?	−	−	−	−
Goré		+++	++	++	+	−	−	−	−
Ehrlich		+++	+++	++	+	+	−*	−*	−*
Vanillin		+++	+++	++	+	+?	−	−	−
Salkowski		+	+	?	−	−	−	−	−

Negative, weak, fair, and strong tests are indicated, by −, +, ++, or +++, respectively.

* Confusing colors appeared in these tubes.

† Oxalic acid tests were held at 37°C. Incubation for a period longer than four days failed to bring out positive tests in any greater dilutions.

TABLE 2

Results of the various tests applied to known dilutions of indol in a killed broth culture

		DILUTIONS OF INDOL PER CUBIC CENTIMETER OF BROTH CULTURE							CONTROL
		0.1 mgm.	0.01 mgm.	0.005 mgm.	0.002 mgm.	0.001 mgm.	0.0005 mgm.	0.0002 mgm.	
Oxalic acid paper†	24 hours	+	?	−	−	−	−	−	−
	48 hours	++	+	?	−	−	−	−	−
	96 hours	+++	++	+	−	−	−	−	−
Goré		+++	++	+	+	−	−	−	−
Ehrlich		+++	+++	++	+	−?*	−*	−*	−*
Vanillin		+++	+++	++	+	?	−	−	−
Salkowski		+	+	−?	−	−	−	−	−

Negative, weak, fair, and strong tests are indicated by −, +, ++, or +++, respectively.

* Confusing colors appeared in these tubes.

† Oxalic acid tests were held at 37°C. Incubation for a period longer than four days failed to bring out positive tests in any greater dilutions.

(1) 2 per cent solution of Difco pepton; (2) 2 per cent Witte pepton, (3) 2 per cent solution of aminoids (50 per cent of total nitrogen as amino nitrogen) and, (4) a casein digest as prepared

by Kulp and Rettger (1924). In a general way the results here corroborated those previously obtained in that the oxalic acid

TABLE 3

Results of various indol tests in different media

METHOD OF TESTING	MEDIUM USED	BACT. COLI		CONTROLS	
		Strain 1	Strain 2	*Bact. cloacae*	Sterile medium
Oxalic acid	Difco pepton, 2 per cent	0	+	0	0
	Witte pepton, 2 per cent	+?	+	0	0
	Aminoids, 2 per cent	0	+	0	0
	Casein digest	+	++	0	0
Goré	Difco pepton, 2 per cent	++	+++	0	0
	Witte pepton, 2 per cent	++	+++	0	0
	Aminoids, 2 per cent	+	+++	0	0
	Casein digest	+++	+++	0	0
Ehrlich	Difco pepton, 2 per cent	+	+++	0*	0*
	Witte pepton, 2 per cent	+++	+++	0*	0*
	Aminoids, 2 per cent	+	+++	0*	0*
	Casein digest	+++	+++	0*	0*
Vanillin	Difco pepton, 2 per cent	++	+++	0†	0†
	Witte pepton, 2 per cent	++	+++	0†	0†
	Aminoids, 2 per cent	++	+++	0	0
	Casein digest	++	+++	0	0
Salkowski	Difco pepton, 2 per cent	++	++	0	0
	Witte pepton, 2 per cent	++	++	0	0
	Aminoids, 2 per cent	0	++	0	0
	Casein digest	+	++	0	0

In this series of tests all tubes were incubated at 37°C. for 48 hours.

Negative, weak, fair and strong tests are indicated by 0, +, ++, and +++, respectively.

* Upon use of the Ehrlich reagent, confusing colors appeared in the uninoculated medium and in the indol-negative cultures. These were usually a lavender and could be distinguished from the reddish purple or deep rose tint produced by contact with indol. The same lavender shade appeared along with the positive tests in many of the indol-positive cultures.

† Confusing colors, other than that of the positive test, appeared frequently in these cases.

test is apparently not as delicate as several of the others, for occasionally a weakly positive culture gave negative results,

particularly when grown in a medium poor in tryptophane. This point is illustrated in table 3 which is representative of other similar experiments. Here *Bact. coli* 1 usually gave a weaker test, by any of the methods, than did no. 2 which was a vigorous indol producer. This is especially noticeable with the Difco pepton and aminoid solutions and it is here that the oxalic acid test gave negative results upon several occasions. On the other hand, the oxalic acid test is apparently reliable in that false positive results were never secured when applied to a rather long list of indol negative cultures and to tubes of sterile media. in addition to those shown in the table. We have also applied the oxalic acid test and the Goré test to cultures grown on agar slants with results essentially similar to those secured by the use of liquid media.

When using the oxalic acid papers with indol-producing cultures it was noticed that the pink color tended to fade after several days if the papers were left in the tube. The loss of color first appeared at the bottom and edges of the paper and gradually spread. This same phenomenon was mentioned by Holman and Gonzales (1923) who suggested that it might be due to the influence of ammonia. It would seem to be caused by some volatile product of bacterial metabolism since in our work it was not observed in the tubes of sterile medium to which indol had been added. In such tubes the pink color gradually changed to an orange brown tint upon long standing, but did not fade. The fading does not seriously interfere with reading of the tests.

The above results may be compared with those obtained by several other investigators. Pittaluga (1909) states that the oxalic acid test gave a positive result after three days with a 1 to 200,000 dilution of indol and that the color even appeared at times in dilutions as high as 1 part per million. Zipfel (1912) found the Ehrlich-Böhme test to give positive results in dilutions as high as 1 to 4,000,000 in aqueous solutions of indol, but in a pepton solution no test was recorded higher than 1 part per million. The Salkowski test was sensitive to 1 part per million in aqueous solution and to 1 part in 400,000 in broth. He also employed oxalic acid paper and although he does not state the

delicacy of this test, he regards it as a simple and valuable control to other tests. Malone and Goré (1921) compared the various methods of applying the Salkowski and the Ehrlich tests but did not use the oxalic acid test. With certain modifications of the Ehrlich test and with the Goré test, they obtained positive results in somewhat higher dilutions of indol than we have been able to do in the present study. They found the nitroso-indol reaction, or Salkowski test, to be very unsatisfactory as ordinarily performed, a conclusion with which we are in complete agreement. Fellers and Clough (1925) have recently published an extensive review of the various methods, in which they found the Ehrlich reagent to be the most delicate and satisfactory. The Goré modification was not employed in their work. The oxalic acid test gave less satisfactory results than several of the more commonly used methods. To increase the delicacy and reliability of the Ehrlich reagent they suggest distillation of the culture, followed by extraction of the distillate and application of the test reagent. By this procedure quantitative results may be secured and they state it is accurate to 1 part in 25,000,000.

In comparing the merits of the oxalic acid test with those of the other procedures employed in our work it is evident that the test is not as delicate as several of the others, notably those which make use of the Ehrlich reagent or vanillin. However, the specificity of both the Ehrlich reagent and vanillin has been questioned and if added directly to the culture medium they may react with phenolic and other compounds in addition to indol. Throughout our work the oxalic acid papers have never shown confusing colors or false positive tests and, in addition, they may be used for detecting indol production on solid media. Although these advantages are somewhat offset by the lack of sensitiveness, it should be noted that in preparing cultures for indol tests the oxalic acid papers may be employed without destruction of the culture so that any other method may then be applied to the same tube.

Of the several indol tests employed in our work, we are inclined to favor the Goré method as the most satisfactory which

may be used where large numbers of cultures are to be examined. It possesses most of the advantages of the oxalic acid method and in addition it is more delicate and the color of the positive test is deeper and more striking than the pink tint of the oxalic acid papers. In our hands the Goré test has never yielded the confusing colors or false positive tests that are secured when the reagents are added directly to the culture, and at the same time appears to be as delicate under these conditions. Also, it has given pronounced and striking positive tests when applied to agar slant cultures of indol-producing organisms, even when a pepton of low tryptophane content was used in the medium.

The method of testing for indol recently recommended by Fellers and Clough (1925) evidently is more delicate than any of the foregoing since they state that it is accurate to 1 part in 25,000,000. While such a method is undoubtedly very useful in detecting and estimating small quantities of indol, the length of the procedure (distillation, ether extraction of the distillate, washing the ether extract and subsequent evaporation of ether) would seem to constitute a decided disadvantage when studying a large number of cultures. It is here that the Goré method would appear to find its greatest usefulness since it may be applied readily to large numbers of cultures, whether grown in liquid or solid media. It has also been found very satisfactory in connection with the study of different bacterial types in classroom work.

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